IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.:	**PENN-0749**
Inventors:	**Muzykantov et al.**
Serial No.:	**09/762,023**
Filing Date:	**June 28, 2001**
Examiner:	**Haddad, Maher M.**
Customer No.:	**26259**
Group Art Unit:	**1644**
Confirmation No.:	**7329**
Title:	**Targeting and Prolonging Association of Drugs to the Luminal Surface of the Pulmonary Vascular Endothelial Cells**

Declaration by Dr. Vladimir Muzykantov

I, Vladimir Muzykantov hereby declare:

1. I am a co-inventor of the above-referenced patent application.

2. I received a degree as a Doctor of Medicine (Internal Medicine) in 1980 from the First Moscow Medical School and a Ph.D. in Biochemistry from the Russian National Cardiology Research Center in 1985. I worked in this Institution in Moscow as a researcher from 1980 to 1993. In 1993 I moved to the University of Pennsylvania where I am still employed in a standing Faculty position of Tenured Associate Professor of Pharmacology and Medicine and secondary appointments in the Institutes of Medical Engineering, Environmental Medicine and Bioengineering Graduate Group. For two decades, my professional career has been focused on research of new drug delivery systems in cardiovascular systems, in particular immunotargeting of therapeutics to the pulmonary vascular endothelium.

2. I am one of the leading world experts in the field of immunotargeting to the pulmonary endothelium having published over 50 peer-reviewed papers and 10 reviews and chapters in this field. I have been invited to talk on this subject by many universities in the United States and Western Europe, as well as several Gordon Research Conferences and many other international conferences. I organized and chaired several symposia on this subject for international conferences of the American Thoracic Society and the Inflammation Research Association. In 2003 I co-edited a book published by Kluwer Science Publishers (Biomedical Aspects of Drug Targeting), in which my review chapter on this subject was included. Since 1995, I was and remain the Principal Investigator of four NIH RO1, two AHA grants and one DOD grant supporting my research in this area. I am requested quite frequently to review papers on this subject for many journals in the USA and abroad. My scientific credentials can be verified in the attached CV.

3. I strongly disagree with the Examiner's statement concerning teachings of Mulligan et al. that it would be immediately apparent to one skilled in the art that the accumulation of the antibody in the pulmonary vasculature reported by Mulligan et al. is due to non-internalization of said antibody. Generally speaking, it is a well-known paradigm that with most studied targets (including tumors, tumor vasculature, brain and peripheral vascular targets), internalizable antibodies show markedly **higher** accumulation *vs* non-internalizable ones. Talking specifically about accumulation in pulmonary vasculature, at the time of this invention (i.e., 1998) among all antibodies with characterized internalizability, only internalizable ones have been known to accumulate in the pulmonary vasculature (see below). In the retrospect, anti-ICAM-1, the subject of our disclosure, represents a rare exception from this rule, but its internalization (more precisely, the lack of thereof) by endothelium had not been fully characterized until 2003 (see below).

4. I am attaching hereto a series of papers published by my laboratory and other research groups that directly state that, in opposite to the Examiner's opinion, accumulation in the pulmonary vasculature is more characteristic of **internalizable** antibodies. Specifically, anti-ACE, anti-caveolar glycoprotein, anti-thrombomodulin and anti-selectin, which have all been demonstrated to accumulate in the pulmonary vasculature after intravascular injection,

bind to internalizable antigens and are internalized by endothelium (see Muzykantov et al. (1996) *Am.J.Physiol.*, 270:L704-713 attached as Exhibit A; McIntosh et al. (2002) *Proc.Natl.Acad.Sci.USA* 99(4):1996-2001 attached as exhibit B; reviewed in Muzykantov, V. (1998) *Pathophysiology*, 5:15-33; Muzykantov, V. (2003) Targeting pulmonary endothelium. "*Biomedical Aspects of Drug Targeting*", Vladimir Muzykantov and Vladimir Torchilin, Eds., Kluwer Academic Publishers, Boston-Dodrecht-London, pages 129-148; and Muro et al. (2004B) *Current Vascular Pharmacology*, 2:281-299 attached as Exhibits C-E, respectively). Furthermore, non-internalizable anti-PECAM antibody did not accumulate in the pulmonary vasculature in animals unless it was converted into internalizable one (see Muzykantov et al. (1999) *Proc.Natl.Acad.Sci.USA*, 96:2379-2384 attached as Exhibit G; and Danilov et al. (2001) *Am.J.Physiol. (Lung)*, 280(6):L1335-347 attached as Exhibit F). The mechanism(s) by which internalization correlates with effective targeting are not fully understood. Most likely, it can be explained by concentration of cell-bound antibody in the intracellular vesicles via recycling membrane antigens, leading to augmentation of total uptake of antibodies by target cells. However, at the time of this invention, for the leading experts in the field and ordinary skilled alike, the fact that anti-ICAM accumulates in the pulmonary vasculature was actually suggestive that this antibody was **internalizable** and thus would **repel** those from development of an antibody conjugate wherein internalization was not desired. In fact, anti-ICAM seems to be one of the first and rare examples of an antibody that accumulates in the pulmonary vasculature **despite** documented lack of internalization (Muro et al. "*Methods in Molecular Biology. Bioconjugation Protocols: Strategies and Methods*", C.M.Niemeyer, Ed., Humana Press, Totowa, NJ, Chapter 2, pages 21-36; Muro et al. (2003) *J Cell Sci.*, 116:1599-1609; and Murciano et al. (2003) *Blood*, 101:3977-1984, attached as Exhibits H-J, respectively). This is the essence of the present invention, which could not be predicted based on the previous knowledge.

5. Further, with respect to the Bowes reference, this reference teaches about totally different matter of subject, i.e., administration of two separate proteins (anti-ICAM and tPA), which act independently. In contrast, our invention describes a new matter of subject, i.e., tPA conjugated with anti-ICAM in a way that these two entities now became a new, single entity with features different from those of both individual components. Those with

ordinary skill of the art would understand that this makes teaching of Bowes irrelevant.

I hereby declare that all statements herein of our own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or by imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application, any patent issuing there upon, or any patent to which this verified statement is directed.

12/09/04
DATE

Vladimir Muzykantov

University of Pennsylvania - School of Medicine

Curriculum Vitae

Vladimir R.Muzykantov, M.D., Ph.D.

Home Address: 101 White Pine Court, Warwick, PA 18974

Office Address: Institute for Environmental Medicine (IFEM), One John Morgan Building, 36th Street and Hamilton Walk, Philadelphia, PA 19104-6068

Citizenship: Naturalized US Citizen

Education:

1974-1980	M.D.	First School of Medicine, Moscow, Russia (Internal Medicine)
1980-1985	Ph.D.	Russian Cardiology Research Center, RCRC, Moscow (Biochemistry)

Professional Training, Post-Doctoral and Research Fellowships:

1976-1979	Work-study student, Academy of Medical Sciences, Enzymology Lab, Moscow
1979-1980	Work-study student, Laboratory of Immunology, RCRC, Moscow
1982-1985	Graduate Student, Biochemistry, USSR Academy of Medical Science, Moscow
1992	Visiting Scientist, Department of Biochemistry, University of Virginia
1993-1995	Will Rogers Memorial Fund Pulmonary Research Fellow, IFEM

Military Service: Not Applicable

Research and Faculty Appointments:

1980-1982	Res.Assistant, Lab. Immunomorphology, Institute of Experimental Cardiology (IEC), Russian Cardiology Research Center (RCRC), Moscow, Russia
1982-1984	Sr.Res.Assistant, Lab.Immunomorphology, IEC, RCRC, Moscow
1894-1987	Jr.Investigator, Lab. Immunomorphology, IEC, RCRC, Moscow
1987-1990	Investigator, Lab.Molecular Endocrinology, IEC, RCRC, Moscow
1990-1993	Sr.Investigator, Department of Biochemistry, IEC, RCRC, Moscow
1993-1994	Sr.Research Fellow, IFEM, University of Pennsylvania, Philadelphia
1994-1996	Research Associate, IFEM, UPENN
1996-	Sr.Investigator, IFEM, UPENN
1997-1998	Research Assistant Professor, Department of Pharmacology, UPENN
1999-2003	Assistant Professor, Department of Pharmacology, UPENN
2003-	Associate Professor (Tenured), Department of Pharmacology, UPENN
2003-	Associate Professor (Adjunct), Department of Medicine, UPENN

Hospital and Administrative Appointments:

Not Applicable

Speciality Certification and Licensure:

Not Applicable

Awards, Honors and Membership in Honorary Societies:

1986	USSR National Young Investigator Award in Pharmacology, 1st Prize
1989	Young Investigator Award, The World Congress of Angiology, Rome, Italy
1990	Young Investigator Award, 16th LH Gray Conference on Tumor Vasculature, Manchester, UK
1992	International Union of Biochemistry Wood Award, Visiting Scientist, Department of Biochemistry, University of Virginia
1992	International Society of Immunology Travel Award, 8th International Congress of Immunology, Budapest, Hungary
1996-2000	Established Investigator, National American Heart Association
2000	The Pulmonary Circulation Assembley Highlight at ATS International Symposium: "Intracellular immunotargeting of active reporter enzyme to endothelium *in vivo*" (05/09/00, Toronto, Canada)
2000	Pulmedica-Preis 2000, First Prize of the German Society of Pulmonary Medicine for the project "Cell-selective intracellular delivery of a foreign enzyme to endothelium *in vivo* using vascular immunotargeting" (presented by Dr. R.Wiewrodt)
2001-2004	Bugher Stroke Award, National American Heart Association
2001	A metal plaque from National American Heart Association "In recognition of outstanding efforts in research fighting the heart disease and stroke".
2001-2002	American Thoracic Scociety, Planning Committee of the National Pulmonary Circulation Assembley, Member
02/10/03	"The Scientist of the Week", Association for Eradication of Heart Attack (AEHA).
09/12/03	Keynote Speaker, 4th Annual Colloqium "Cellular and Molecular Biomechanics", University of Virginia, Charlottesville, VA
04/16/04	University of Pennsylvania, Magister of Arts *Honoris Causa*

Membership in Professional and Scientific Societies:

1993-	Inflammation Research Association, Member
1995-	The American Heart Association, Member
1999-	The American Physiological Society, Member
1999-	The American Thoracic Society, Member

Editorial Positions and Activity (Ad Hoc Reviewer):

1997-	Journal of Applied Physiology
	International Journal of Cancer
	Free Radical Biology and Medicine
	The Israel Science Foundation
1998-	Natural Sciences and Engineering Research Council of Canada

	Biochim.Biophys.Acta (Biomembranes)
1999-	Thrombosis Research
	Antioxidant and Signal Transduction
2000-	General Pharmacology of Vascular System
	Nature Medicine
2001-	Am.J.Physiol., Lung Molecular and Cellular Physiology
2002-	Am.J.Physiol., Cellular Physiology
	J.Pharm.Exp.Therapeutics
2003-	Am.J.Resp.Cell.Mol.Biol.
	Molecular Cancer Therapeutics
2004-	Exp.Lung.Research
	J.Gene Medicine
	Am.J.Resp.Crit.Care Med.
	Br.J.Pharmacology
	Blood
	Microvascular Research
	Expert Opinion on Drug Delivery

Invited Lectures and Seminars (since moved to the USA in 1993):

11/10/94	TNO-Gaubius Institute, Leiden University, Leiden, The Netherlands
02/03/97	Department of Biochemistry, Virginia University, Charlottesville, VA
03/03/97	Department of Physiology, Louisiana State University, Shreveport, LA
11/21/97	Centocor, Malvern, PA
05/12/98	Department of Neonatology, St.Christopher Hospital, Allegheny University, Philadelphia
06/19/98	Smith-Kline Beecham, King of Prussia, PA
07/20/98	Invited Speaker, Summer FASEB Conference "Physiology and Pathophysiology of Circulation", Portland, Oregon
05/09/00	Invited Speaker, ATS International Conference, Toronto, Canada
06/21/00	Invited Speaker, International Conference on Avidin Technologies, Banff, Canada
02/21/01	Eccles Institute of Human Genetics, University of Utah, Salt Lake City, Utah
07/13/01	Institute of Biomembranes, Utrecht University, Utrecht, the Netherlands
10/16/01	Institute for Medical Engineering, University of Pennsylvania
01/18/02	Department of Neonatology, Children's Hospital of Philadelphia
02/06/02	Department of Pharmacology, University of Illinois in Chicago
01/25/02	Invited Discussant, Transatlantic Airway Conference "Oxidants/Antioxidants" Lucerne, Switzerland
02/24/02	Invited Speaker, **Gordon Research Conference** on Drug Delivery, Ventura, CA
05/24/02	Invited Speaker, ATS International Conference, Atlanta, GA
07/10/02	Invited Speaker, International Symposium "Reactive Oxygen Species", St.Petersburg, Russia,
09/31/02	University of Cologne, Germany
10/01/02	University of Mainz, Germany
10/03/02	Urbino University, Italy
12/16/02	Centocor, Radnor, PA

02/24/03 Department of Radiology, Harvard/MGH, Boston, MA
03/05/03 Invited Speaker, International Symposium of Controlled Release and Advanced Drug Delivery Systems, Salt Lake City, Utah
04/04/03 Department of Pharmacogenetics, Pittsburgh University, PA
06/17/03 EluSys Therapeutic Systems, Pine Brook, NJ
06/24/03 Department of Cardiology, Emory University School of Medicine, Atlanta, GA
07/30/03 Department of Pharmaceutical Sciences, University of Nebraska, Omaha
08/10/03 Invited Speaker, 6th World Congress on Inflammation (International Association Inflammation Societies Congress, IAIS), August 2-6th, Vancouver, Canada
09/12/03 Keynote Speaker, 4th Annual Colloqium "Cellular and Molecular Biomechanics", University of Virginia, Charlottesville, VA
10/02/03 CardioPulmonary Research Institute, Winthrop University Hospital, SUNY at Stony Brook School of Medicine, Mineola, NY
10/07/03 Sol Sherry Thrombosis Research Center, Temple University, Philadelphia, PA
10/13/03 EluSys Therapeutics Scientific Advisory Board Meeting, Pine Brook, NJ
11/21/03 Department Molecular Cardiology, Cleveland Clinic Foundation, Cleveland, OH
01/19/04 Debiopharm SA, Lausanne, Switzerland
01/21/04 Invited Discussant, Transatlantic Airway Conference "Gene and Drug Therapies of Airway Diseases", Lucerne, Switzerland
02/11/04 Invited Speaker, **Gordon Research Conference** "Oxygen Radicals in Biology", Ventura, CA, February 8-13, 2004
04/10/04 Drexel University, Department of Bioengineering, Philadelphia, PA
08/26/04 Invited Speaker, **Gordon Research Conference** "Endothelial cell phenotypes in health and diseases", Andover, NH, August 22-27, 2004
10/06/04 Department of Physiology, John Hopkins University
10/15/04 Invited Speaker, Annual Red Blood Cell Club Symposium, Columbia, MO
11/03/04 Division of Cardiovascular Medicine, University of Michigan, Ann Arbor, MI
11/16/04 Department of Bioengineering, Ohio University, Athens, OH
12/02/04 Symposium of the University of Pennsylvania Institute for Medical Engineering
01/18/05 Cardiovascular Research Center, MGH-Harvard, Boston, MA
01/28/05 Department of Physiology, Case Western Reserve University

Organizing Role in Scientific Meetings:

1991, International Advisory Committee Member, 2nd International Conference on Eicosanoids and Bioactive Lipids in Cancer and Inflammation, Berlin, FRG, 1991
2000, Chariman, American Thoracic Society International Conference, Symposium "Targeting of drugs and genes to the pulmonary endothelium", Toronto, May 5-10, 2000
2002, Chairman, American Thoracic Society International Conference, Symposium "Focus on the pulmonary endothelium", Atlanta, GA May 18-24, 2002
2003, Chairman, Symposium on Targeted Drug Delivery, 6th World Congress on Inflammation (International Association Inflammation Societies Congress, IAIS), August 2-6th, Vancouver, Canada
2004, Discussion Leader, Gordon Conference on Drug Delivery, Big Sky, MT, 09/08/04

Academic Commttees at Previous Institutions and at the University of Pennsylvania:

1986-1991	Russian National Cardiology Research Center, Young Scientists Society Steering Committee, Vice-Chairman (1998-1991)
1996-	Faculty, NRSA in Lung Cellular and Molecular Biology, IFEM, UPENN
1998-2003	Steering Committee for SCOR in Acute Lung Injury, Member
1999-	Faculty, NRSA in Lung Immunology, Pulmonary Medicine Division, UPENN
1999-	Institute of Medical Engineering, UPENN, Member
2002-	Center for Cancer Pharmacology, UPENN, Member
2002	School of Medicine Strategic Planning Committee (Cardiovascular), Member
2003-2004	Department of Path. &Lab. Medicine Academic Review Committee, Member
2004-	Faculty, NRSA in Cardiovascular Biology, IME
2004-	Curriculum Committee, Pharmacology Graduate Group, Member
2004-	Membership Committee, Pharmacology Graduate Group, Member
2004-	Dean's Standing Committee of Chairs and Directors, Substitute Member (IFEM)

Teaching Activity:

University of PENN School of Medicine lectures:

1999-2000	Pharmacology, Module I: Targeted drug delivery
1999-present	Cardiology, Module II: Drugs affecting myocardial ischemia
2001-present	Frontiers in Medicine: Biomedical aspects of targeted drug delivery
2003-present	Frontiers in Medicine: Drug targeting to tumors

Faculty Member of the Graduate Groups at PENN

2001-present	Pharmacology, School of Medicine
2002-present	Bioengineering, School of Engineering and Applied Sciences,

Course Director, Graduate Courses in Pharmacology:

2001-present	PHRM-570: Principles in cardiovascular biology
2002-2004	PHRM-590: Areas of research in pharmacology at PENN

Lectures in Pharmacology Graduate Courses

2001-present	PHRM-650 (seminar): Targeted drug delivery
2001-present	PHRM-570 Course: Targeted delivery of drugs in cardiovascular system
2002-present	PHRM-570 Course: Ischemia-Reperfusion and vascular oxidative stress
2002-present	PHRM-560 Course: Targeted delivery of drugs to tumors
2005-	PHRM-623 Course: Emerging methods of drug delivery

Post-Doctoral Fellows and Research Associates, PENN:

1993-1997	E.Atochina, M.D., Ph.D.
1995-1997	I.Balyasnikova, Ph.D.,
1997-2003	J.Murciano, Ph.D.
2001-present	S.Muro-Galinda, Ph.D.
2001-present	K.Gunguly, Ph.D.

2002-present	V.Shuvaev, Ph.D.
2003-present	S.Zaitsev, Ph.D.
2004-present	C.Danielyan, Ph.D.

NRSA Post-Doctoral Fellows, PENN:

1999-2000	L.Koniaris, M.D.
1999-2001	T.D.Sweitzer, Ph.D.
2002-2004	T.Dziubla, Ph.D.

Visiting Foreign Fellows and Students

1994, 1995	J-C.Murciano, Pre-Doctoral Fellow, Spain
1998-2002	R.Wiewrodt, M.D., Post-Doctoral Pulmonary Fellow, FRG, (with S.Albelda)
1999, 2001	A.Sherpereel, M.D., Post-Doctoral Pulmonary Fellow, France, (with S.Albelda)
2002	A.Panchenko, summer student from St.Petersburg Medical Academy, Russia
2002-2003	E.Berk, Visiting Undergraduate Student, the Netherlands

Work-study, part-time and summer students at University of Pennsylvania:

1994-1997	Ankur Luther Joshi (U.PENN)
2001-2004	Tanya Krasik (U.PENN)
2001-present	Christina Gayevsky (U.PENN)
2003-present	Anu Thomas (Jefferson University)
2004-present	Jeremy Pick (U.PENN)

Graduate Students:

1989-1993	Ph.D. Thesis Advisor, Alla B.Zaltzman, IEC, Moscow, Ph.D. Thesis: "Modification of red blood cells with biotin and avidin" (Biochemistry, 1993, USSR National Cardiology Center, Moscow). Present position: Post-doctoral fellow, Medical College of Wales, Cardiff, UK
1988-2000	Advising experimental studies of F.Branco, 1997-2000, Ph.D. Thesis "Endothelial cell culture for intracellular oxidative stress", (Temple University, Physiology, 05/19/2000)
2004-present	Ph.D. Thesis Advisor, Weining Qui, (Bioengineering)

Rotation Graduate Students

2002	Robinson, Mary (Pharmacology, Summer Semester)
2003	Colon, Franchesca (Pharmacology, Fall Semester)
2003	Fuentes, Rudy (Pharmacology, Fall Semester)
2004	Weining Qui, (Bioengineering, Spring Semester)
2004	Bisen Ding (Pharmacology, Summer Semester)
2004	Armen Karamajan (Pharmacology, Fall Semester)
2004	Eric Simone (Bioengineering, Fall Semester)
2004	Julie Zurpina (Bioengineering, Fall Semester)

Ph.D. Thesis Committees at PENN:

2000-2004	Committee Member for A.Omolola Eniola (Department of Bioengineering, SEAS) "Characterization of biodegradable targeted drug delivery vehicle"
2001-2003	Committee Member for Jeffrey Guinrich, IME (Bioengineering)
2003-2004	Committee Member for Yuqin Hui, Department of Pharmacology
2003-present	Committee Chair for Wenying Jang, Department of Pharmacology
2004-present	Committee Member for Mary Robinson, Department of Pharmacology

Grants and Extramural Funding

Please note that the grant system has been introduced in the former USSR in 1990; hence no grant support is listed prior to 1991. Monetary conversion of USSR grants is not available.

Completed

Principal Investigator:

- 1991-1993, USSR Department of Health, Grant-in-Aid: "Immunotargeting of glucose oxidase to the pulmonary endothelium."
- 1992-1993, USSR Academy of Sciences Coincil on Enzymology Research Grant: "Conjugation of enzymes with ACE antibody".
- 1995-1997, **National AHA Grant-in-Aid**: "Immunotargeting of antioxidant enzymes to the pulmonary endothelium." ($88,000 direct cost)
- 1998-1999, University of Pennsylvania Research Foundation Grant: "Conjugation of plasminogen activators to the carrier red blood cells" (total $20,000, direct cost)
- 1997-1999, **National American Lung Association Research Grant**: "Targeting of plasminogen activators to the pulmonary vasculature" (direct cost: $50,000)
- 1988, **NIH HLBI RO1:** "Regulation of oxidants in the pulmonary endothelium". **(This grant that got proirity score 145, a percentile 2.7% at the first submission 10/01/97, has been relinquished in order to accept NIH SCOR in Acute Lung Injury and it continued now as an RO1** HL073940-01-A1, **see below).**
- 1999-2000, Co-PI, The University of Pennsylvania Research Foundation Grant: "Prevention of cardiopulmonary bypass induced pulmonary dysfunction by immunotargeting of antioxidant enzymes to the pulmonary endothelium" (Dr. W.Gaynor, PI, direct cost $20,000)
- 1997-2000, **The National AHA Established Investigator Grant**: "Protection of the vascular endothelium against oxidative insults by specific targeting of antioxidants" (direct cost $300,000)
- 1999-2000, Sponsored Research Agreement (SRA), Centocor: "Evaluation of the anti-thrombotic profile of Reo-Pro and Retavase in animal model of pulmonary microembolism" (direct cost: $38,000, total cost: $60,000)
- 1998-2003, Project Leader, **Project 4, NIH HLBI SCOR in Acute Lung Injury**: "Augmentation of antioxidant defenses by immunotargeting", Project 4, direct cost $705,788, total cost $1,115,146 (A.B.Fisher, Program Director, 1 P50 HL60290-01)

Co-Investigator on grants of other PIs:

- 1994-1998, co-Investigator, NIH HLBI RO1 Grant: "Ishemia-Reperfusion Injury to Lung" (A.B.Fisher, PI).
- 1999-2004, co-Investigator, NIH HLBI RO1 "Structure-function analysis of the human urokinase receptor" (D.Cines, PI)

Sponsor or preceptor:

- 1998-1999, Dr. J.Murciano, a Recipient, NATO Research Postdoctoral Fellowship "Selective targeting of fibrinolytic agents to the vasculature", (stipend $25,000)
- 1999-2001, Dr. J.C.Murciano, a Recipient, Spanish National Fellowship Foundation, Postdoctoral Research Grant "Delivery of plasminogen activators to the pulmonary vasculature" (total stipend $40,000)

- 2000-2001, Dr. T.Sweitzer, PI, PENN IHGT Pilot Grant, "Nuclear targeting of non-viral nucleid acid vectors" (direct cost: $45,000)
- 2001-2002, Dr. S.Muro, a Recipient, Fundacion Ramon Areces Fellowship (Spain) for a Post-Doctoral fellowship abroad the country, Section: Natural Sciences: "Immunotargeting of conjugates to the endothelium: applicability for drug, enzyme and gene therapy" (total stipend $48,000)

Currently funded:

Principal Investigator

- 2001-2005, **National AHA Bugher Stroke Award** #0170035N, "Thromboprophylaxis of cerebral microembolism", total cost $400,000
- 2001-2005, **NIHLBI, RO1 HL66442-01** "Thromboprophylaxis in the surgical settings", direct cost: $700,000, total cost $1,109,000
- 2002-2006, **Department of Defense Research Grant DAMD 17-02-1-0197** (PR 012262) "Targeting of drugs to ICAM for treatment of acute lung injury", direct cost $829,683, total cost 1,283,287
- 2002-2006, **NIHLBI RO-1 HL71175-01** "Drug Targeting to ICAM-1", direct cost: $900,000, total cost: $1,344,600
- 2003-2005, **Sponsored Research Agreement with EluSys Therapeutics, Inc.**,: "Synthesis and characterization of anti-CR1/tPA conjugate" (direct cost: $169,144, total cost: $260,000)
- 2004-2008, **NHLBI RO1 HL073940-01-A1** "Augmentation of antioxidant defenses by immunotargeting" (score 156, percentile 6.1%, funding decision pending, projected time: direct cost: $1,000,000, total cost: $1,585,250).
- 2004-2009, **NHLBI RO1 HL078785-01** "Nanocarriers for delivery of antioxidants to endothelium" (direct cost $1,000,000, total cost $1,585,250)

Sponsor:

- 2004-2009, Dr. J-C.Murciano, a Recepient, Fundacion Ramon y Cajal Award "Targeting of anti-thrombotic agents in the vasculature" (A five-year fellowship in Cardiovascular Research Center, Madrid, Spain)
- 2004-2008, Dr. S.Muro, National American Heart Association Scientist Development Grant "Endothelium-Targeted Enzyme Replacement Therapy for Lysosomal Storage Disorders", $260,000 direct costs
- 2004, Dr. T.Dziubla, "New Methoxy-Poly(Ethylene Glycol)-Poly(Lactone) Di-Block Copolymers For The Synthesis Of Nanoparticles With Acid Accelerated Degradation Profiles", NTI Seed Grant ($30,000)

Pending:

Principal Investigator and Lead Investigator

- 2005-2010, Project Leader, Project 4 "Nanocarriers for delivery of antioxidants to endothelium" (NHLBI PO1 "Reactive oxygen species and antioxidants in ALI", A.B.Fisher, Program Director, submitted 02/01/04, an alternative RO1 HL078785-01)
- 2005-1010, Lead Investigator, UO1, Center of Excellence in Nanotechnology (P.F.Davies, PI) "Cardiovascular targets of nano-worm micelles"

Bibliography (* indicates papers in which VM is a corresponding senior author):

I. Research Publications, peer reviewed:

1. G.Samokhin, M.Smirnov, **V.Muzykantov**, S.Domogatsky and V.Smirnov (1983) Red blood cell targeting to collagen-coated surfaces. *FEBS Lett.*, 154:257-261
2. M.Smirnov, G.Samokhin, **V.Muzykantov**, G.Idelson, S.Domogatsky and V.Smirnov (1983) Type I and III collagen as a possible target for drug delivery to the injured sites of vascular bed. *Biochem.Biophys.Res.Commun.*, 116:99-105
3. G.Samokhin, M.Smirnov, **V.Muzykantov**, S.Domogatsky and V.Smirnov (1984) Effect of flow rate and blood cellular elements on the efficiency of targeting to collagen-coated surfaces. *J.Appl.Biochem.*, 6:70-75
4. A.Klibanov, **V.Muzykantov**, N.Ivanov and V.Torchilin (1985) Evaluation of quantitative parameters of the interection of antibody-bearing liposomes with target antigens. *Anal.Biochem.*, 150:25-257
5. S.Domogatsky, G.Idelson, O.Merzlikina, **V.Muzykantov,** A.Roodin, B.Shekhonin and V.Rukosuev (1985) Preparation and characterization of antibodies to collagen. *Immunology* (Moscow, in Russian) 3:78
6. B.Shekhonin, S.Domogatsky, **V.Muzykantov**, G.Idelson and V.Rukosuev (1985) Distribution of type I, III, IV and V collagen in normal and atherosclerotic human arterialwall. *Coll.Rel.Res.*, 5:355-368
7. **V.Muzykantov***, G.Samokhin, M.Smirnov and S.Domogatsky (1985) Hemolytic complement activity assay in microtitration plates. *J.Appl.Biochem.*, 7:223-227
8. **V.Muzykantov***, D.Sakharov, M.Smirnov S.Domogatsky and G.Samokhin (1985) Targeting of enzyme immobilized on erythrocyte membrane to collagen-coated surfaces. *FEBS Lett.*, 182:62-66
9. M.Glukhova, S.Domogatsky, A.Kabakov, **V.Muzykantov,** O.Ornatsky, D.Sakharov, M.Frid and V.Smirnov (1986) Red blood cell targeting to smoth muscle cell. *FEBS Lett.*, 198:155-158
10. V.Smirnov, S.Domogatsky, V.Dolgov, V.Hvatov, A.Klibanov, V.Koteliansky, **V.Muzykantov**, V.Repin, G.Samokhin, B.Shekhonin, M.Smirnov, B.Torchilin, D.Sviridov and E.Chazov (1986) Carrier-directed targeting of liposomes and erythrocytes to denuded areas of vessel wall. *Proc.Natl.Acad. Sci.USA,* 83:6603-6607
11. **V.Muzykantov***, D.Sakharov, M.Smirnov, G,Samokhin and V.Smirnov (1986) Immunotargeting of erythrocyte-bound streptokinase provides local lysis of fibrin clot. *Biochim.Biophys.Acta*, 884:355-363
12. G.Samokhin, M.Smirnov, **V.Muzykantov** and S.Domogatsky (1986) Red blood cells as a carrier for drug targeting to injured sites of vasculature. *Bull.Natl.Cardiol.Res.Ctr.USSR* (Moscow, in Russian), 1:84-89
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1. **V.Muzykantov**, E.Barnathan, S.Danilov. "Immunotargeting of plasminogen activators to the pulmonary endothelium", US Patent 5,653,979, issued 08/05/1997

2. **V.Muzykantov**, A.Higazi, J.Murciano, D.Cines. "Compositions and methods for treatment of uncontrolled formation of intravascular fibrin clots", US Patent 6,488,927 B2, issued 12/03/2002

3. **V.Muzykantov** and S.Albelda. "Enhancement of intracellular delivery and tissue targeting of drugs and genes", Pending US Patent filed by PENN March 10, 1998
   Australian Patent # 753303, issued 09/17/2002

4. **V.Muzykantov**, J.Murciano, D.N.Granger. "Targeting and prolonging association of drugs to the luminal surface of the pulmonary vascular endothelial cells", Pending US Patent filed by PENN August, 4, 1998
   (Australian Patent #754117, issued 02/27/2003)

5. D.Cines, J.Murciano, **V.Muzykantov**, M.Nakada. "Methods and compositions for the treatment of pulmonary embolism using a IIb/IIIa receptor antagonist and a thrombolityc", Pending US Patent filed by Centocor, 2001

6. **V.Muzykantov**, A.Higazi, J.Murciano, D.Cines, R.Taylor "Compositions and methods for treatment of uncontrolled formation of intravascular fibrin clots" (Pending divisional application PENN-0831, filed in US Patent Office 11/03/1999)

7. **V.Muzykantov**, J.Murciano, D.Cines. "Methods for selective dissolution of the nascent intravascular blood clots avoiding lysis of pre-existing haemostatic clots" (Pending continuation-in-part US Patent application filed 07/01/2003)

8. T.Dziubla and **V.Muzykantov** "Nanoencapsulated antioxidant enzymes for the treatment of sub-acute/chronic oxidative stress" (Disclosure Q 3349 filed by CTT as a provisional US patent application 11/05/03)

Extracurricular Activities: Writing songs (lyrics and music) since 1977.
Composed more than 150 songs, including 10 songs on verses of other poets.

Book of lyrics for the authored songs "Dances on a fire" (Moscow, 1993)
Numerous songs published in the Russian "Author's songs" almanachs in 1991-2001.
Five selected songs included in the academic edition of "The Antology of Russian Author Songs", Moscow, 2002 (D.A.Sukharev, Editor).

Recorded two laser CD albums with solo singing of his songs:
"The Mirror" (New York, 2001)
"Cosmopolitan" (Philadelphia, 2003)

Six songs from "The Mirror" and "Cosmopolitan" have been included in compilation CD albums:
"Russian Bards in America" (New York, 2002)
"Guitar goes around" (Moscow, 2003)
"Remember - it happened at a seaside..." (Moscow, 2004)

A dedicated CD album "15 songs of Vladimir Muzykantov" (Moscow, 2004, producer L.Cheboksarova)
Features selected Muzykantov's songs performed by famous Russian bards and actors V.Zolotukhin, E.Kamburova, V.Mischuk, A.Kozlovsky, A.Ivashenko, D.Bogdanov, B.Kiner, M.Zitrinyak, G.Dudkina, O.Mityaev, E.Frolova, V.Egorov, L.Sergeev and other.

More than 10 songs have been recorded by professional singers on their CDs:
Lidya Cheboksarova "People of Dry Ways" (Moscow, 1998)
Lidya Chboksarova "A Pantomima" (Moscow, 2000)
Leon Pozen "Winter Waltz" (Seattle, 2001)
Vadim and Valiry Mischuk "Our favorite songs" (Moscow, 2001)
Lidya Cheboksarova "The Third" (Moscow, 2003)

PENN-693

Endothelial cells internalize monoclonal antibody to angiotensin-converting enzyme

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Muzykantov, Vladimir R., Elena N. Atochina, Alice Kuo, Elliott S. Barnathan, Kathy Notarfrancesco, Henry Shuman, Chandra Dodia, and Aron B. Fisher. Endothelial cells internalize monoclonal antibody to angiotensin-converting enzyme. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L704–L713, 1996.—We investigated the fate of MAb 9B9, a monoclonal antibody to angiotensin-converting enzyme (ACE), which binds to endothelium both in vitro and in vivo. Using cultured human umbilical vein endothelial cells (HUVEC) and isolated perfused rat lungs (IPL), we demonstrated specific and saturable binding of ^{125}I-labeled MAb 9B9 at 4°C [affinity constant (K_d) = 20–50 nM, maximal number of binding sites (B_{max}) = 1.5–3.0 × 10^5 sites/cell]. When ^{125}I-MAb 9B9 was bound to HUVEC at 37°C, only 40% of cell-associated radioactivity was acid elutable, suggesting antibody internalization. This was confirmed by finding that *1*) the amount of MAb 9B9 uptake at 37°C was higher than at 4°C both in HUVEC and IPL; *2*) binding of ^{125}I-labeled streptavidin with HUVEC and IPL pretreated with biotinylated MAb 9B9 (b-MAb 9B9) was diminished in a temperature- and time-dependent fashion at 37°C; and *3*) b-MAb 9B9 bound to HUVEC at 37°C was found intracellularly by ultrastructural analysis using streptavidin gold. Intracellular ^{125}I-MAb 9B9 was found in microsomal fractions of lung homogenate from IPL and after intravenous (iv) injections in rats. Degradation of internalized MAb 9B9 was minimal, since >90% of cell-associated ^{125}I label remained precipitable by trichloracetic acid in HUVEC, IPL, and in vivo. Autoradiography of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lung homogenates made as late as several days after iv injections of ^{125}I-MAb 9B9 in rats demonstrated a predominant band above 140 kDa. These data indicate that endothelial cells either in vitro or in vivo internalize the ACE ligand MAb 9B9 without significant intracellular degradation. Therefore MAb 9B9 may be useful for selective intracellular delivery of drugs to the pulmonary vascular endothelium after systemic administration.

drug targeting; vascular biology; lung; pulmonary vasculature; angiotensin-converting enzyme turnover; rats

ANGIOTENSIN-CONVERTING ENZYME (ACE) is a carboxydipeptidase localized in the plasma membrane of the vascular endothelium and some other cell types (10). ACE converts angiotensin I into a potent vasopressor, angiotensin II, and inactivates bradykinin, substance P, and other vasoactive and neurotransmitter peptides (28). Because of its location, ACE could serve as a target molecule for drug targeting to the pulmonary vascular endothelium. MAb 9B9, a monoclonal antibody to ACE, accumulates selectively in the lungs of various animal species after systemic administration (9). Various compounds conjugated with MAb 9B9 accumulate selectively in the lungs of laboratory animals after systemic injection (8, 20, 23). MAb 9B9 does not fix complement, does not inhibit ACE enzymatic activity, and does not induce endothelial injury either in vitro or in vivo (5). Thus MAb 9B9 appears to be a safe and specific carrier for drug targeting to the pulmonary endothelium (21).

In the present study we address the possibility of ACE internalization in the vascular endothelium. This issue is important in terms of vascular biology because of the key role of ACE in the regulation of vascular tone (10), as well as in terms of drug targeting, because internalization would be important for the action of some drugs (27). However, very little is known about internalization of ACE or ACE ligands, ACE recycling in the endothelial cells, or the effect of ACE ligands on ACE internalization. ACE is a transmembrane glycoprotein that is not considered to possess receptor or signaling function; the only accepted pathway of ACE metabolism in the plasma membrane is its shedding to the extracellular medium (10, 26). Several previous publications have addressed turnover of membrane-associated ACE, albeit indirectly. For example, Johnson and Erdos (15) in early work demonstrated that endothelial cells in culture do not bind or internalize radiolabeled ACE. Some authors consider this as evidence against ACE turnover in the plasma membrane (24), although uptake of exogenous ACE may be irrelevant to membrane turnover and recycling of cellular ACE. One recent study suggested that endothelial cells do not internalize monoclonal antibodies to ACE, because cytochalasin B failed to inhibit antibody-induced enhancement of ACE shedding from cultured cells (18). This result, however, may be irrelevant to ACE turnover in the plasma membrane, since ACE shedding and internalization may have different mechanisms. The possibility of antibody uptake by endothelial cells has not yet been evaluated, and there are no reported studies of internalization of ACE ligands, ACE substrates, ACE inhibitors, or ACE antibodies. Therefore, issues about membrane turnover of ACE (constitutive or ligand-induced) and internalization of ACE ligands still need to be elucidated.

To clarify the potential of anti-ACE MAb 9B9 as an affinity carrier for intracellular targeting to the pulmonary endothelium, we have studied its interaction with cultured human umbilical vein endothelial cells (HUVEC) and with rat pulmonary endothelium both in vivo and in the isolated perfused rat lung (IPL). Our results demonstrate that *1*) endothelial cells internalize MAb 9B9, *2*) MAb 9B9 does not undergo significant degradation within the endothelial cell after internalization, and *3*) subcellular fractionation of lung after MAb 9B9

uptake indicates accumulation of the antibody in the microsomal fraction.

MATERIALS AND METHODS

Streptavidin and 6-biotinylaminocaproic acid *N*-hydroxysuccinimide ester (BxNHS) were obtained from Calbiochem (San Diego, CA); iodogen was obtained from Pierce (Rockford, IL); Na^{125}I was obtained from Amersham (Arlington Heights, IL); streptavidin-gold, dimethyl formamide (DMF), fluorescein isothiocyanate (FITC), trichloroacetic acid (TCA), and normal mouse immunoglobulin (Ig)G were obtained from Sigma (St. Louis, MO); fatty acid-free bovine serum albumin (BSA) was obtained from Boehringer-Mannheim (Indianapolis, IN); cell media and supplementary materials were obtained from GIBCO Life Technology (Gaithersburg, MD).

Labeling of antibody. MAb 9B9 and control mouse IgG were biotinylated with BxNHS at biotin-to-IgG molar ratio of 10 (b-MAb 9B9) as described (22). This previous study confirmed that b-MAb 9B9 displays functional activity similar to that of nonbiotinylated MAb 9B9 (22). MAb 9B9 and IgG were labeled with ^{125}I using iodogen-coated tubes according to the manufacturer's recommendations. Specific radioactivity was in the range of 0.3–1.0 × 10^6 counts per minute (cpm)/μg of protein; 98% of radioactivity was TCA precipitable. MAb 9B9 and IgG were labeled with FITC (FITC-to-IgG molar ratio of 50) as described (2). Excess label (biotin, isotope, or FITC) was removed by Sephadex G-25 gel-filtration.

Interaction of radiolabeled MAb 9B9 with cultured human endothelial cells. We used HUVEC obtained and cultured as described previously (14). Medium 199 (M199) containing 20% heat-inactivated fetal calf serum, growth factors, heparin, and antibiotics (M199 supplemented) was used for culture and incubation procedures. Cells were passaged two to four times, subcultured onto 96-well microtiter plates using trypsin-EDTA mixture, and allowed to reach confluence for 24 h. For an estimation of cellular binding, ^{125}I-MAb 9B9 or control ^{125}I-IgG was added to washed cells in M199 containing 0.2% BSA and incubated for the indicated time at 4°C. After cells were washed, cells were detached using trypsin-EDTA mixture, and cell-associated radioactivity was estimated in a gamma-counter. To estimate the specificity of binding, nonlabeled MAb 9B9 was coincubated in wells with ^{125}I-MAb 9B9. Specific binding of MAb 9B9 was calculated by subtraction of nonspecific binding (i.e., ^{125}I-MAb 9B9 binding in the presence of nonlabeled MAb 9B9) from the total binding (i.e., ^{125}I-MAb 9B9 binding in the absence of nonlabeled MAb 9B9).

To determine uptake of MAb 9B9 by the endothelium, confluent cells were incubated with ^{125}I-MAb 9B9 for the indicated time at 37°C. Radioactivity bound to the cell surface was released by incubation with acidic buffer, as described previously (14). Briefly, after cells were washed of nonbound radioactivity, cells were incubated with 50 mM glycine in 100 mM NaCl solution, pH 2.5 (15 min at 20°C). There was no cell detachment after this procedure, according to examination by light microscopy. After collection of eluates from glycine-treated cells, cells were detached by incubation with trypsin-EDTA. Glycine-eluted radioactivity (i.e., radioactivity of glycine eluates) and cell-associated radioactivity (i.e., radioactivity of trypsin-EDTA extracts) were estimated in a gamma counter and added to calculate total radioactivity. Percent internalization was calculated as % = (total radioactivity − glycine eluted) × 100/total radioactivity.

To determine degradation of antibody and detachment of radiolabel from the antibody, we used a standard assay of TCA-soluble radiolabel described previously (22). Briefly, 50% TCA was added to surface-associated radiolabel (glycine eluates) and to cell-associated radiolabel (trypsin-EDTA extracts) in the presence of 10 mg/ml BSA as a carrier protein (final TCA concentration was 10%). After 1-h incubation at 4°C, samples were centrifuged at 2,000 rpm for 10 min and radioactivity in the pellet and supernatants was estimated. The percentage of TCA-soluble radiolabel (i.e., percent degradation) was calculated as % = (supernatant radioactivity) × 100/total radioactivity.

To determine kinetics of antibody dissociation and release from HUVEC to the medium, confluent cells were incubated with 1 μg of ^{125}I-MAb 9B9 for 2 h at 37°C. After elimination of nonbound antibody, cells were incubated for indicated time at 37°C and then radioactivity in cell medium and in cell-associated fractions was determined. Assay of TCA-soluble radiolabel in the medium was performed as described above.

Interaction of ^{125}I-labeled streptavidin with cultured endothelial cells treated with b-MAb 9B9. Streptavidin was radiolabeled with ^{125}I and its activity was assessed as described in our previous study (20). To estimate binding of ^{125}I-streptavidin, confluent cells were incubated with b-MAb 9B9 or b-IgG for 2 h at 4°C. After elimination of nonbound immunoglobulins, cells were incubated for 40 min at 4°C with ^{125}I-streptavidin, washed, detached from plastic with trypsin-EDTA mixture, and cell-associated radioactivity was determined. In a second series of experiments, cells were incubated with b-MAb 9B9 for 3 h at 37°C or 4°C and, after washing, binding of ^{125}I-streptavidin was determined at 4°C. In a third experiment, cells were incubated with b-MAb 9B9 for 2 h at 4°C. After cells were washed, cells were incubated for indicated time at 37°C to allow antibody internalization, and then binding of ^{125}I-streptavidin was determined.

Visualization of internalization of b-MAb 9B9 by cultured endothelial cells using streptavidin-gold. HUVEC cultured in 24-well Multiwell plates were incubated with b-MAb 9B9 (1 μg/well in 0.3 ml of M199, 3 h at 37°C), washed, and permeabilized by 20-min incubation at 37°C with 0.05% Tween-20 [added in 1 ml of Dulbecco's phosphate-buffered saline (PBS), pH 7.4 per well]. Cells in control wells were not permeabilized (incubation with Dulbecco's PBS only). Cells were washed with Dulbecco's PBS and incubated for 1 h at room temperature with 10 nm streptavidin-gold particles. After cells were washed of nonbound streptavidin-gold particles, cells were fixed with 4% paraformaldehyde in PBS. Cell morphology was examined by phase-contrast microscopy, while streptavidin-gold particles were visualized by microscopy in bright field.

Perfusion of isolated rat lungs. Sprague-Dawley male rats (Charles River Breeding Laboratories, Kingston, NY), weighing 200–300 g, were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg). Anesthetized rats were ventilated through a cannula placed at the level of the tracheal carina by the humidified gas mixture of 95% air-5% CO_2. Ventilation was performed using a rodent ventilator (Harvard Apparatus, Millis, MA) at 60 cycles/min, 2-ml tidal volume, and 2 cmH_2O end-expiratory pressure. The thorax was opened, the pulmonary artery was cannulated via the right ventricle, and the left atrium was transected for the collection and recirculation of the perfusate. Lungs were isolated, cleared of blood in a single pass perfusion for 5 min, and placed in the isolated lung perfusion apparatus. Lungs were perfused with Krebs-Ringer bicarbonate (KRB) buffer, containing 10 mM glucose and 3% (wt/vol) fatty acid-free BSA (pH 7.4), at a flow rate of 10 ml/min using a peristaltic pump (Harvard Apparatus) (13).

To study the interaction of MAb 9B9 with IPL, 1 μg of ^{125}I-MAb 9B9 or ^{125}I-IgG was added to the perfusate. After recirculation with antibody-containing perfusate for indicated time at room temperature, the lungs were perfused in a

single-pass mode for 5 min with KRB-BSA that did not contain antibody. Lungs were removed from the chamber, rinsed with saline, weighed, and radioactivity was estimated in a gamma-counter. In a separate experiment, nonlabeled MAb 9B9 was added to perfusate with 1 μg of ^{125}I-MAb 9B9, and tissue uptake of radiolabel was assessed as described above.

To visualize the uptake of circulating MAb 9B9 in IPL, 50 μg of FITC-labeled MAb 9B9 or FITC-labeled IgG was added to the perfusate. After a recirculating perfusion of 1 h at 37°C, the lungs were perfused with KRB-BSA in a single-pass mode for 10 min. Then lungs were perfused for 5 min at room temperature with 4% paraformaldehyde in 0.1 M cacodylate buffer. After further fixation in a vial with the same fixative for 3 h, lungs were washed with 0.1 M cacodylate buffer, dehydrated in graded ethanol, embedded in PolyBed 812 epoxy, cut into 2-μm sections, mounted on glass slides, and examined by confocal microscopy.

To estimate the disappearance of MAb 9B9 from the luminal surface of the pulmonary endothelium, we determined binding of ^{125}I-streptavidin in IPL after perfusion of b-MAb 9B9. For this purpose, lungs were perfused in a recirculating mode with KRB-BSA containing 10 μg of b-MAb 9B9 or b-IgG for 40 min at 37°C. Then the lungs were perfused in a single-pass mode for 5 min with KRB-BSA that did not contain antibody. Lungs were then perfused with KRB-BSA by recirculation at 37°C. At indicated times, ^{125}I-streptavidin was added to the perfusate and perfused by recirculation for 20 min at 37°C. Finally, nonbound streptavidin was removed by perfusion with KRB-BSA in a single-pass mode for 5 min, and radioactivity in the lung tissue was determined.

To determine the kinetics of antibody dissociation and release from the lung tissue, lungs were perfused with 1 μg of ^{125}I-labeled b-MAb 9B9 for 40 min at 37°C. Excess antibody was removed by perfusion with KRB-BSA in a single-pass mode for 5 min. Lungs were then perfused with KRB-BSA by recirculation at 37°C. At indicated times, lungs were perfused with KRB-BSA in a single-pass mode for 5 min, and radioactivity in the lung tissue was determined.

Distribution of MAb 9B9 in subcellular fractions of the lung tissue homogenate. Lungs were perfused with 10 μg of radiolabeled MAb 9B9 for 1.5 h at 37°C, as described above. Nonbound MAb 9B9 was removed by perfusion with KRB-BSA in a single-pass mode for 10 min. At the end of perfusion, lungs were immediately frozen in liquid nitrogen and stored at −70°C. Lung tissue homogenate and subcellular fractions were prepared by sucrose gradient centrifugation as described previously (12). The purity of subcellular fractions was characterized with assays for NADPH-cytochrome *c* reductase (25) and 5′-nucleotidase (1). Protein concentration in fractions was determined by Bio-Rad micro-protein assay using Coomassie blue and bovine IgG as standard, according to the manufacturer's recommendation. Radioactivity in the fractions of the homogenates of lungs perfused with ^{125}I-MAb 9B9 was determined in a gamma-counter and expressed as counts per minute per milligram of protein. To characterize radiolabeled proteins in the lung tissue after perfusion of ^{125}I-MAb 9B9, fractions of homogenate were analyzed by nonreducing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), electroblotting to nitrocellulose, and autoradiography of nitrocellulose filters (30). To obtain detectable radioactivity levels in the filters after electrophoresis and blotting, we loaded 200–400 μg of protein/lane of 1.5 mm 9% gel with 3% stacking gel.

Biodistribution and pulmonary accumulation of radiolabeled or b-MAb 9B9 in rats. ^{125}I-MAb 9B9 (5 μg/rat) was injected via tail vein in rats under intraperitoneal pentobarbital anesthesia (50 mg/rat). At indicated times after injection, animals were exsanguinated and internal organs and blood were collected. Biodistribution of radioactivity was determined by counting of iodine isotope in gamma-counter in rinsed organs and in blood. Analysis of ^{125}I-MAb 9B9 in lung tissue from rats after injection in vivo (estimates of radioactivity, TCA-soluble radiolabel, and SDS-PAGE of fractions of the lung homogenate) was performed as described above for IPL experiments.

RESULTS

MAb 9B9 binding to endothelial cells in cell culture, in isolated perfused rat lung, and in rat lungs after in vivo administration. In the first series of experiments we characterized MAb 9B9 binding in three models: cultured endothelial cells (Fig. 1*A*), isolated perfused rat lungs (Fig. 1*B*), and after intravenous injection in rats (Fig. 1*C*). An immunospecificity index [ISI; defined as the ratio of uptake of ^{125}I-MAb 9B9 vs. ^{125}I-IgG (21)] was calculated to compare the specificity of antibody binding in these models quantitatively. In HUVEC ^{125}I-MAb 9B9 binds to cells with ISI equal to 10 (Fig. 1*A*). Nonlabeled MAb 9B9 inhibits binding of ^{125}I-MAb 9B9 by 80%, confirming the specificity of MAb 9B9 binding to HUVEC. In IPL, ^{125}I-MAb 9B9 accumulates in rat lung tissue with ISI equal to 40 (Fig. 1*B*). After intravenous injection, ^{125}I-MAb 9B9 accumulates selectively in the rat lungs with pulmonary ISI (calculated by dividing the lung-to-blood ratio of ^{125}I-MAb 9B9 by that of ^{125}I-IgG) equal to 20. In a separate experiment we perfused FITC-labeled MAb 9B9 in isolated rat lungs. This procedure led to specific (as compared with FITC-IgG) pulmonary uptake of fluorescent label (Fig. 2). Confocal microscopy of tissue sections after FITC-MAb 9B9 perfusion in rat lung shows a bright fluorescent labeling of endothelial cells (Fig. 2*A*). Thus presented data confirm specific binding of MAb 9B9 to endothelial cells in culture and in rat lungs.

Interaction of ^{125}I-MAb 9B9 with cultured human endothelial cells. To inhibit uptake of cell-bound antibody by cells, we studied the binding of MAb 9B9 to HUVEC at 4°C. ^{125}I-MAb 9B9 demonstrates saturable binding to HUVEC at 4°C, inhibitable by addition of nonlabeled MAb 9B9 (Fig. 3). In the experiment shown in Fig. 3, affinity constant (K_d) and maximal number of binding sites (B_{max}) estimated by Scatchard plot were 40 nM and 1.8×10^5 antibody molecules/cell. For preparations of ^{125}I-MAb 9B9 used in HUVEC (second to fourth passages), K_d varied in the range 20–50 nM and B_{max} varied in the range of $1.3–3.2 \times 10^5$ sites/cell (data not shown).

We next studied whether HUVEC internalize MAb 9B9 by comparing uptake at 37°C and 4°C. At equilibrium, which was reached in 150–200 min, the cells took up 492 ± 16.7 vs. 93 ± 16.5 pg ^{125}I-MAb 9B9/well at 37°C and 4°C, respectively, indicating active cellular uptake of cell-associated antibody. Using a glycine-elution assay, we estimated the fraction of cell-associated radiolabel that could be released into the medium after incubation of HUVEC with ^{125}I-MAb 9B9.

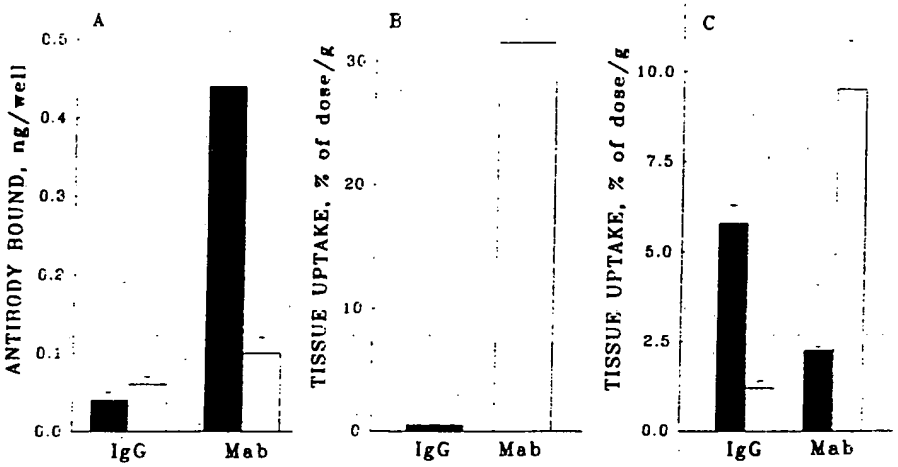

Fig. 1. Comparison of the specificity of binding of ^{125}I-labeled MAb 9B9 with endothelial cells in three experimental systems. *A*: cultured human umbilical vein endothelial cells (HUVEC) were incubated with 0.5 μg of radiolabeled immunoglobulin (IgG (*left* or MAb 9B9 (Mab) (*right*) for 3 h at 4°C. After washing, radioactivity in wells was determined (solid bars). Open bars, cells were incubated with 0.5 μg of radiolabeled MAb 9B9 or IgG in the presence of 30 μg of nonlabeled MAb 9B9 or IgG. Data are presented as bound antibody/well, means ± SD, n = 3. *B*: isolated perfused lung (IPL). Fresh isolated rat lungs were perfused for 1 h with recirculating perfusate containing 1 μg of radiolabeled IgG (solid bar) or MAb 9B9 (open bar). After perfusion, nonbound radioactivity was eliminated by a single-pass perfusion of antibody-free or IgG-free perfusate for 5 min. Data are presented as a percentage of perfusate radioactivity accumulated in the lung/g of tissue, means ± SD, n = 3. *C*: in vivo administration. Normal rats were injected through tail vein with 0.5 ml of PBS-BSA containing 1 μg of radiolabeled IgG (*left*) or MAb 9B9 (*right*). One hour after injection animals were killed and radioactivity in the blood (solid bars) and in the lungs (open bars) was determined. Data are presented as a percentage of injected dose/g of tissue, means ± SD, n = 3.

Incubation of HUVEC with ^{125}I-MAb 9B9 at 37°C led to time-dependent reduction of radiolabel released by acid treatment. After incubation for 120 min, 52.3 ± 5.9% vs. 97.5 ± 0.5% of the cell-bound radioactivity was released by glycine buffer at 37°C and 4°C, respectively (Fig. 4). Therefore, there is a time-dependent disappearance of ^{125}I-MAb 9B9 from the cell surface at physiological temperature.

We also studied kinetics of ^{125}I release into the medium from HUVEC-treated ^{125}I-MAb 9B9 at 37°C. During 90 min of cell incubation at 37°C in the antibody-free medium, only 6.7 ± 0.8% of cell-associated radioactivity was released to the medium, while 93 ± 4% remained associated with cells. Of the released radioactivity, 76 ± 15% was TCA-soluble. This value is equal to ~4% of total cell-associated radioactivity.

It is noteworthy that the percent of internalization was independent of the dose of ^{125}I-MAb 9B9 incubated with HUVEC at 37°C; over the range of 75–1,000 ng of antibody/well, 55–56% of the antibody was internalized. The percent of TCA-soluble radiolabel from the membrane and intracellular compartments was independent of dose in this range (1–4% and 5–7%, respectively).

Internalization of b-MAb 9B9 by cultured endothelial cells. As a second approach to estimate MAb 9B9 internalization by endothelial cells, we studied binding of ^{125}I-streptavidin to cells pretreated with b-MAb 9B9.

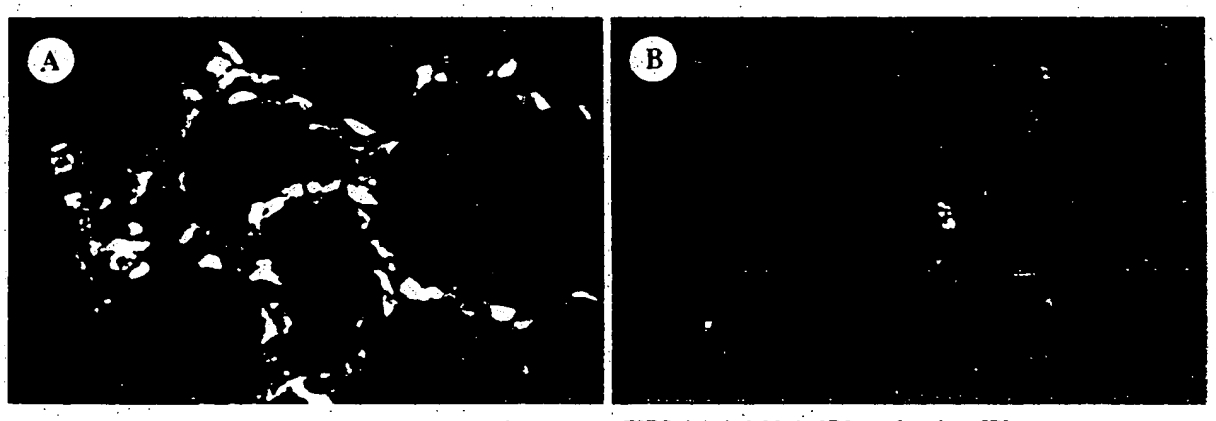

Fig. 2. Visualization of tissue uptake of fluorescein isothiocyanate (FITC)-labeled MAb 9B9 perfused in IPL. Confocal fluorescent microscopy of rat lungs perfused with FITC-conjugated MAb 9B9 (*A*) or FITC-conjugated IgG (*B*). FITC-conjugates (50 μg) were perfused in IPL for 1 h at 37°C. After 10 min of single-pass perfusion with conjugate-free perfusate, lungs were perfused for 5 min with fixative solution.

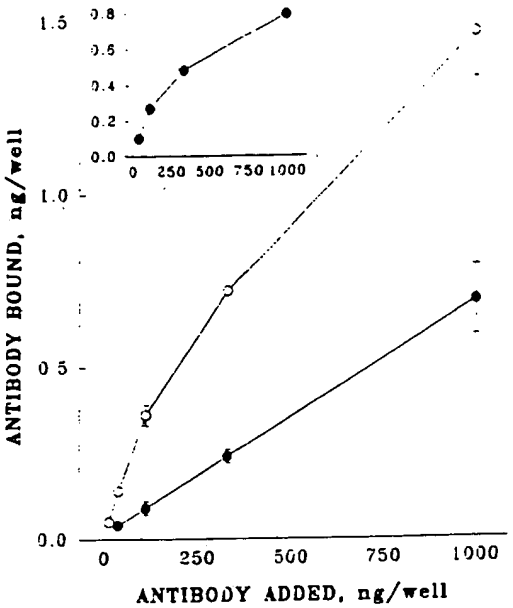

Fig. 3. Binding of radiolabeled MAb 9B9 with HUVEC. HUVEC were incubated for 3 h at 4°C with radiolabeled MAb 9B9 (○) or with radiolabeled MAb 9B9 in the presence of nonlabeled MAb 9B9 (30 μg/well, ●). Means are ±SD, $n = 3$. *Inset*: calculated specific binding of radiolabeled MAb 9B9 to HUVEC at 4°C; axes are the same as for the major figure.

^{125}I-streptavidin binds specifically to HUVEC preincubated at 4°C with b-MAb 9B9 (Fig. 5*A*). ^{125}I-streptavidin binding to cells preincubated with b-MAb 9B9 at 4°C was even higher than its binding to cells preincubated with b-MAb 9B9 at 37°C (Fig. 5*B*, *right*). In contrast, cellular uptake of ^{125}I-labeled b-MAb 9B9 was higher at 37°C than at 4°C (Fig. 5*B*, *left*). This result indicates that most of the b-MAb 9B9 associated with cells at 37°C is inaccessible to streptavidin in the medium. We used ^{125}I-streptavidin to evaluate the kinetics of b-MAb 9B9 disappearance from the surface of endothelial cells incubated at 37°C after pretreatment with b-MAb 9B9 at 4°C. Compared with the value immediately after pretreatment with b-MAb 9B9, streptavidin binding after a chase of 1 or 2 h was reduced to 30% and 10% of initial level, respectively. Therefore, endothelial cells incubated at 37°C internalized b-MAb 9B9.

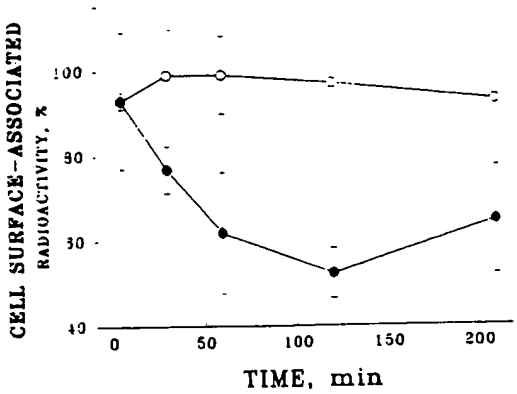

Fig. 4. Internalization of radiolabeled MAb 9B9 by human endothelial cells. HUVEC were incubated with 200 ng of radiolabeled MAb 9B9 for indicated time at 4°C (○) or at 37°C (●). After washing of nonbound antibody, membrane-associated radioactivity was assessed by elution with 50 mM glycine buffer, pH 2.5, while internalized radioactivity was assessed in trypsin-EDTA eluates. Data are presented as surface-associated radioactivity calculated as %total radioactivity (see text). Results are means ± SD, $n = 3$.

Intracellular uptake of b-MAb 9B9 was visualized in HUVEC by microscopy using streptavidin-gold particles (Fig. 6). Cells were preincubated with b-MAb 9B9 at 37°C, permeabilized after washing of nonbound b-MAb 9B9, and incubated with streptavidin-gold particles at 4°C. This procedure led to accumulation of streptavidin-gold particles in endothelial cells (Fig. 6*B*). Cellular uptake of streptavidin-gold particles was dramatically reduced when the permeabilization step was omitted (Fig. 6*D*), indicating that the streptavidin-gold particles shown in Fig. 6*B* were intracellular.

Binding, internalization, and tissue degradation of MAb 9B9 in perfused rat lung. To extend our study to a more physiological model, we characterized uptake of ^{125}I-MAb 9B9 in isolated perfused lungs (Fig. 7). The half-time of ^{125}I-MAb 9B9 uptake in IPL was ~30 min (Fig. 7*A*), while control ^{125}I-IgG did not accumulate in the isolated rat lungs. ^{125}I-MAb 9B9 specifically interacts with endothelial ACE in IPL, since addition of nonlabeled MAb 9B9 to perfusate inhibits binding of ^{125}I-MAb 9B9 in a dose-dependent fashion (not shown). Figure 7*B* shows that ^{125}I-MAb 9B9 uptake in IPL is saturable. Linearization of this binding curve in Scatchard plot indicates B_{max} of 14 μg of MAb 9B9 (i.e., ~5×10^{13} MAb 9B9 molecules) per gram of the lung tissue. To calculate the B_{max} per cell for MAb 9B9 in the rat lungs, we estimated from previously published morphometric data that rat lung (net wt 1.5 g) contains ~4×10^8 endothelial cells (4). Thus, K_d and B_{max} for ^{125}I-MAb 9B9 in perfused lungs were ~18 nM and 2.5×10^5 sites/cell.

To study the internalization of MAb 9B9 in IPL, we compared the uptake of ^{125}I-MAb 9B9 in lungs where the perfusate temperature was maintained at 37°C or at 8°C. Decrease in the temperature of the perfusate led to a marked reduction of accumulation of radioactivity in the lung tissue [21.9 ± 0.9 % injected dose (ID)/g vs. 36.9 ± 6.7 %ID/g, $P < 0.05$], indicating temperature-dependent uptake of ^{125}I-MAb 9B9 by the pulmonary endothelium (Fig. 8*A*). Second, we estimated disappearance of b-MAb 9B9 from the pulmonary vasculature using ^{125}I-streptavidin. After perfusion of ^{125}I-b-MAb 9B9 at 37°C and prolonged perfusion of antibody-free solution at 37°C, radioactivity was retained in the lung tissue (Fig. 8*B*, closed circles). In contrast, after perfusion of b-MAb 9B9 at 37°C, tissue uptake of ^{125}I-streptavidin was decreased in a time-dependent fashion during perfusion of antibody-free solution at 37°C (Fig. 8*B*, open circles), indicating b-MAb 9B9 disappearance from the luminal surface of the pulmonary endothelium.

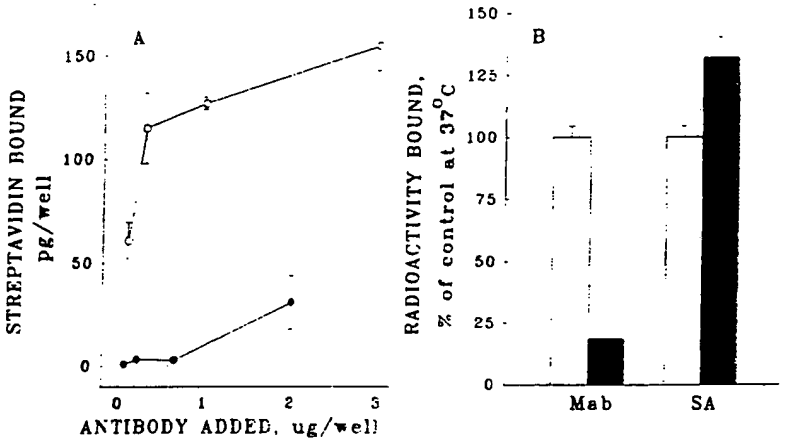

Fig. 5. Interaction of radiolabeled streptavidin with HUVEC pretreated with biotinylated (b-) MAb 9B9. *A*: HUVEC were incubated for 2 h at 4°C with b-MAb 9B9 (○) or with b-IgG (●), washed, and incubated with radiolabeled streptavidin (500 ng/well) for 40 min at 4°C. *B*: influence of temperature on cellular uptake of ^{125}I-labeled b-MAb 9B9 (*left*) or binding of ^{125}I-labeled streptavidin to cells pretreated with b-MAb 9B9 (SA, *right*). HUVEC were incubated with antibody and streptavidin at 37°C (open bars) or at 4°C (solid bars). Data are presented as a percent of binding of radiolabeled preparation at 37°C, means ± SD, $n = 3$.

We characterized the subcellular distribution and evaluated possible degradation of MAb 9B9 in the perfused rat lungs. The microsomal fraction was enriched in radioactivity (5,080 cpm/mg of protein), as compared with initial homogenate (1,703 cpm/mg), plasma membrane fraction (1,840 cpm/mg), and the cytosolic fraction (787 cpm/mg). Radiolabel that was TCA soluble was 4.5% in the lung tissue homogenate, 10% in the cytosolic fraction, 0.1% in the plasma membrane fraction, and 1% in the microsomal fraction.

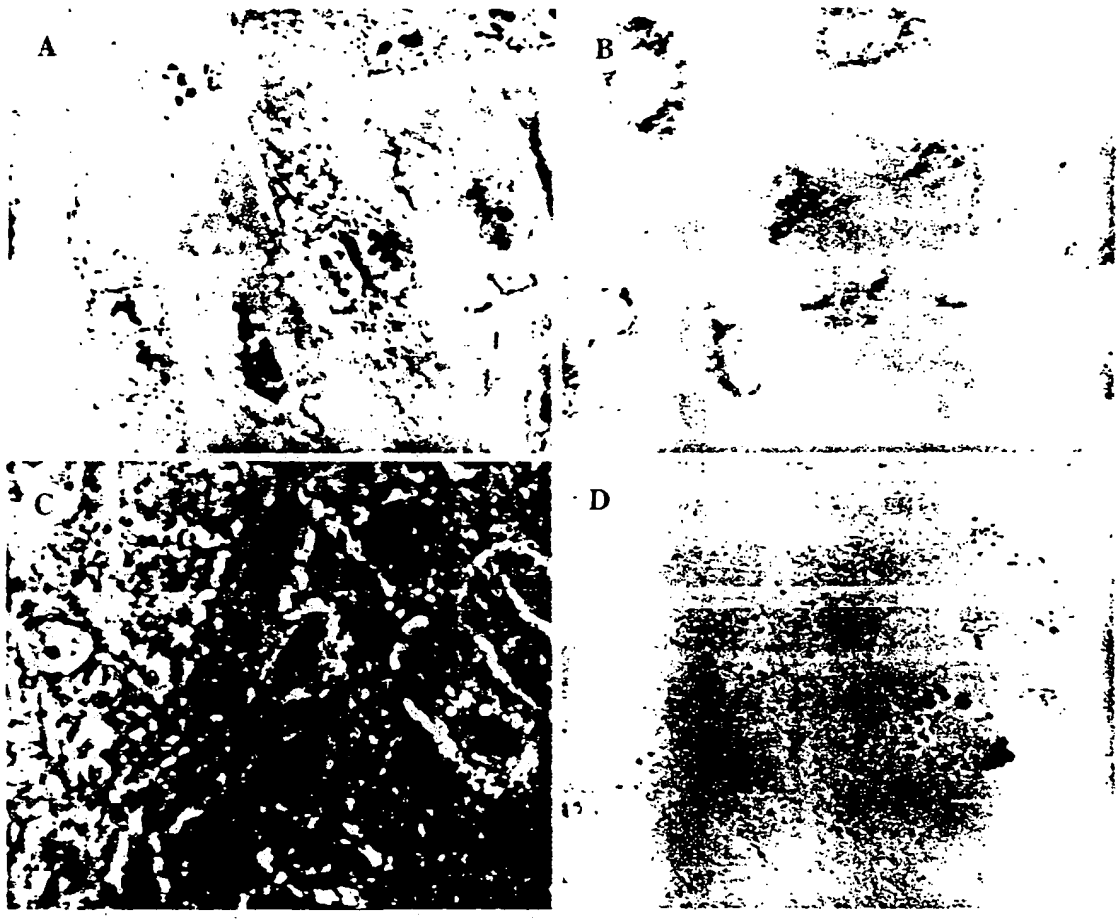

Fig. 6. Binding of streptavidin-gold particles to endothelial cells pretreated with b-MAb 9B9. HUVEC were incubated for 3 h at 37°C with b-MAb 9B9 (1 μg/well), washed, and incubated further for 1 h with streptavidin-gold particles at 4°C. After final washing, cells were fixed by incubation with paraformaldehyde. *A* and *B*: after washing of nonbound b-MAb 9B9. HUVEC were permeabilized by detergent. *C* and *D*: cells were not permeabilized. *A* and *C*, phase contrast; *B* and *D*, visualization of streptavidin-gold particles in bright field.

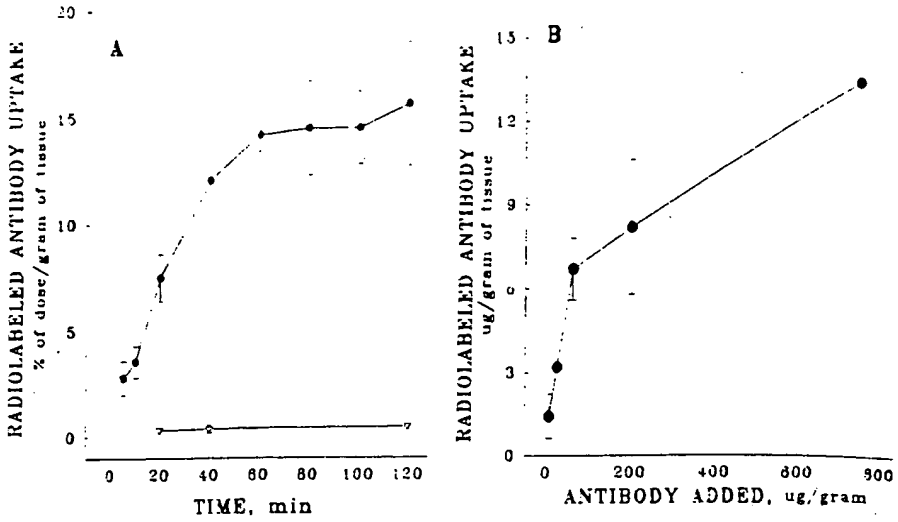

Fig. 7. Specific pulmonary accumulation of radiolabeled MAb 9B9 in isolated perfused rat lungs (IPL, perfusion at room temperature). *A*: kinetics of pulmonary uptake of radiolabeled MAb 9B9 (●) and IgG (▽) in IPL, shown as a percent of perfused radioactivity/g of the lung tissue, means ± SD, $n = 4$. SD values for IgG binding are smaller than symbols. *B*: dose dependence of pulmonary uptake of radiolabeled MAb 9B9 in IPL (40-min perfusion), means ± SD, $n = 3$.

Thus, as in HUVEC, the total percentage of TCA-soluble radiolabel in IPL did not exceed 5–10%. We characterized the radiolabeled protein in fractions of the lung tissue homogenate obtained from IPL perfused with ^{125}I-MAb 9B9 for 1.5 h at 37°C. For this purpose, the fractions were analyzed by autoradiography after nonreducing SDS-PAGE (Fig. 9*A*) and electroblotting to nitrocellulose (Fig. 9*B*). In all fractions, only one

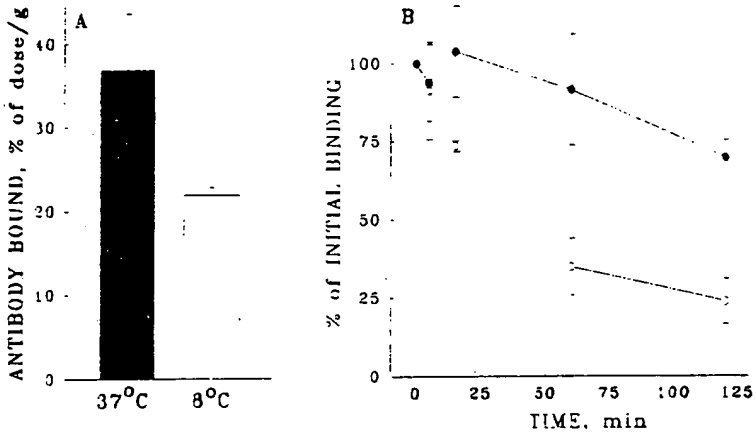

Fig. 8. Tissue uptake of MAb 9B9 in IPL. *A*: comparison of pulmonary uptake of ^{125}I-MAb 9B9 perfused at 37°C (solid bar) and 8°C (open bar). Note that temperature was measured in the perfusate and not in the lung tissue, where it could be higher. Means are ± SD, $n = 3$. *B*: time-dependent reduction of pulmonary uptake of ^{125}I-streptavidin perfused in IPL after perfusion of b-MAb 9B9. (●) Radioactivity in the lung tissue after perfusion of IPL for 40 min with 1 μg ^{125}I-b-MAb 9B9 following 5-min single-pass perfusion with antibody-free perfusate and an additional recirculating perfusion of antibody-free KRB-BSA; time of this perfusion is indicated at *x* axis. (○) Same protocol until the addition of 20-min perfusion of radiolabeled streptavidin and then 5-min single-pass perfusion of streptavidin-free perfusate. Data are presented as a percent of pulmonary uptake of radiolabel with *time 0* at 45 min after the start of perfusion, means ± SD, $n = 3$.

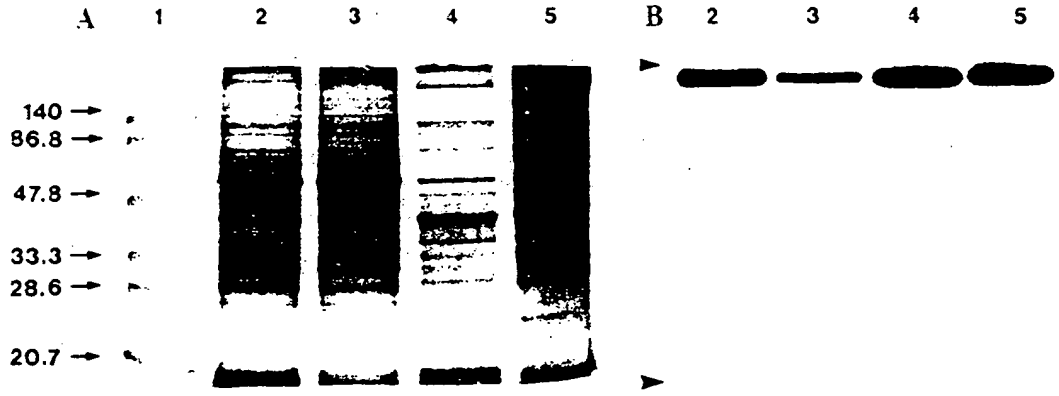

Fig. 9. Characterization of radiolabeled proteins in the rat lung perfused with radiolabeled MAb 9B9. Radiolabeled MAb 9B9 (10 μg) was perfused in IPL as described in Fig. 8 legend. Fractions of lung homogenate were obtained by centrifugation as described in MATERIALS AND METHODS. *A*: Coomassie blue staining of SDS-PAGE of the lung homogenate (*lane 2*), cytosol fraction (*lane 3*), microsomal fraction (*lane 4*), and plasma membrane fraction (*lane 5*). *Lane 1*: calibration standards. *B*: autoradiography of proteins transblotted to nitrocellulose filter from gel shown in *A*. Arrowheads, origin and front of gel.

Table 1. *Distribution of radiolabel in fractions of the lung homogenates 1.5 h after intravenous injection of radiolabeled rat MAb 9B9*

Fraction	Radioactivity, cpm/mg	%TCA-soluble label
Homogenate	3,975	2.9 ± 0.1
Plasma membranes	4,877	0.92 ± 0.04
Microsomes	5,683	1.2 ± 0.1
Cytosol	860	6.1 ± 0.9

TCA-soluble data are means ± SD; $n = 3$. Rat was injected with 10 μg of b-MAb 9B9 in tail vein and killed 1.5 h after injection. Lungs were washed free of blood by perfusion with saline, isolated, and frozen. Lung homogenate and fractions were prepared, and radioactivity and % TCA-soluble radiolabel in fractions were estimated as described in MATERIALS AND METHODS.

radioactive band was found (above 140 kDa). No products of degradation of ^{125}I-MAb 9B9 were detected in any fraction.

Pulmonary distribution and degradation of MAb 9B9 injected in rats. To evaluate the fate of MAb 9B9 in the target organ in vivo, we injected rats intravenously with ^{125}I-MAb 9B9. After intravenous injection, MAb 9B9 accumulated selectively in the rat lungs (lung-to-blood ratio of 14.2), while control nonimmune isotype-matched mouse IgG did not (lung-to-blood ratio of 0.16). Confirming our previous work (9, 21, 23), there was no accumulation of ^{125}I-MAb 9B9 in any organ except the lung (not shown). Table 1 shows distribution of radiolabel in fractions of the lung homogenate 1.5 h after in vivo injection of ^{125}I-MAb 9B9. Confirming the results obtained in IPL, radioactivity was low in the cytosolic fraction (860 cpm/mg protein) and high in the microsomal fraction (5,683 cpm/mg). The percentage of TCA-soluble label was maximal in cytosol and least in plasma membrane fraction, confirming results obtained in HUVEC and IPL. Up to several days after intravenous injection of radiolabeled MAb 9B9 in rats, radioactivity in all fractions of the lung tissue homogenate was associated with a single band comparable with intact IgG, demonstrating the same autoradiographic pattern as obtained after perfusion of ^{125}I-MAb 9B9 in IPL (not shown).

DISCUSSION

In the present work we have studied internalization of anti-ACE MAb 9B9 by vascular endothelial cells. This antibody recognizes a conformational-dependent epitope localized on the extracellular domain of ACE molecule remote from the active site of ACE (7). MAb 9B9 possesses unique potential as an affinity carrier for selective delivery of drugs to the pulmonary endothelium of various animal species, including humans (21). Therefore, the issue of MAb 9B9 internalization is of interest in terms of drug targeting, since intracellular delivery may be beneficial or necessary for action of certain drugs (27).

To trace the interaction of MAb 9B9 with endothelial cells, we have modified MAb 9B9 by three different labels: ^{125}I, FITC, and biotin. Radiolabeled antibody was useful for direct quantitation of cellular binding, uptake, release, and degradation of antibody, as well as for tracing MAb 9B9 in tissue fractions. FITC-labeled MAb 9B9 was used for direct visualization of MAb 9B9 interaction with lung tissue in isolated lung. b-MAb 9B9 was useful to study antibody accessibility from the lumen after binding to endothelium. This combination of tracers allows us to exclude with reasonable certainty *1*) effects of antibody modification on its interaction with ACE or with endothelial cells, and *2*) metabolism of label in the endothelial cells or in the lung.

We have studied MAb 9B9 interaction with endothelial cells in three different models. The cultured human endothelial cell model offers the possibility to study wide variations of experimental conditions (dose, kinetics, temperature) and thus is useful for quantitation of binding, uptake, release, and degradation of ligands. Isolated perfused lung allows study of interaction of antibody with endothelial cells in the whole organ in the absence of systemic effects and provides enough material for isolation of cellular fractions for tracing of antibody trafficking in the tissue. In vivo injection of labeled MAb 9B9 is the most physiological model, providing data on targeting, tissue uptake, distribution, and stability of antibody in the target tissue. Using these models, we were able to compare formal parameters of MAb 9B9 binding in cell culture and in the real target organ. Interestingly, parameters of binding of radiolabeled MAb 9B9 to target cells in both models were similar (Figs. 2 and 6). Thus B_{max} for MAb 9B9 in both models was in the range of $1.3–5.2 \times 10^5$ antibody molecules/cell.

Our data indicating that MAb 9B9 recognizes a variety of endothelial cells correlate well with previous data obtained immunochemically by Danilov and coworkers (6). In sections of human tissues, MAb 9B9 appears to stain endothelial cells in every tissue, as well as staining some types of epithelial and neuroepithelial cells (6). Thus the apparent selective accumulation of MAb 9B9 in the lung reflects the distribution of endothelial cells in the body; specific pulmonary uptake of 30% of injected dose of radiolabeled MAb 9B9 correlates with the estimation that ~30% of the total body vascular endothelium is associated with the pulmonary vasculature (16). Recent immunomorphological studies, however, demonstrated heterogeneity of ACE distribution in the endothelial cells in the various vascular areas (11). Moreover, Ryan and co-authors (29) have demonstrated heterogeneity of ACE distribution in the rat lungs, using single-pass perfusion of radioactive ACE substrates. According to data of Morrell and colleagues (19), precapillary and capillary endothelium express more ACE than venous endothelium in the rat lung. Pulmonary uptake of MAb 9B9 in IPL may, therefore, reflect interaction of MAb 9B9 with a virtual "median endothelial cell" in the pulmonary vasculature, whereas contribution of precapillary and capillary endothelial cells in MAb 9B9 binding may be predominant.

The major finding of this work is a positive answer to the following question: Do endothelial cells internalize MAb 9B9? Our study presents several lines of evidence in support of this conclusion. First, cellular uptake of

^{125}I-labeled MAb 9B9 is higher at 37°C compared with 4°C or 3°C, as demonstrated in both HUVEC and IPL. Second, cell-associated MAb 9B9 becomes inaccessible to removal from the cell surface after incubation at physiological temperature. This has been demonstrated by acid elution of radioactivity in HUVEC after binding of ^{125}I-MAb 9B9, as well as by binding of ^{125}I-streptavidin in HUVEC and in IPL after pretreatment of the target with b-MAb 9B9. Third, intracellular uptake of MAb 9B9 in the vascular endothelium has been demonstrated with streptavidin-gold in HUVEC.

In the present study we did not address the mechanisms of MAb 9B9 internalization by endothelium. Because internalization did not depend on MAb 9B9 concentration, it is unlikely that the mechanism for internalization was antibody-induced cross-linking of ACE in the membrane and cellular uptake of clustered antigen. It is well known that the effectiveness of antigen cross-linking by antibody depends on the antigen-to-antibody ratio for formation of immune complexes that are dose dependent. As an alternative mechanism for MAb 9B9 internalization, we suggest two possibilities. One possibility is that the ACE molecule undergoes constitutive turnover and recycling in the endothelial plasma membrane, like some other endothelial membrane proteins (3, 17). If such constitutive ACE turnover occurs, ACE could serve as a carrier for intracellular transfer of MAb 9B9 bound to the endothelial cell. The second possibility is that ACE itself is not normally internalized, but the interaction of MAb 9B9 with ACE induces internalization of the complex. A recent study demonstrated that binding of some monoclonal antibodies to ACE molecule leads to its conformational alteration (7), and a similar change may occur with MAb 9B9.

While the mechanism of MAb 9B9 internalization by endothelial cells remains to be elucidated, MAb 9B9 internalization by itself is of importance in terms of MAb 9B9-mediated drug targeting to the pulmonary endothelium. From this point of view, the fate of internalized antibody is of particular interest. Our study provides several lines of evidence that the major fraction of internalized MAb 9B9 remains intact after endothelial uptake. First, the TCA-soluble radiolabel after ^{125}I-labeled MAb 9B9 uptake in HUVEC, in fractions of lung homogenate in IPL and after in vivo administration, did not exceed 10%. Second, SDS-PAGE of lung tissue homogenate after injection of ^{125}I-labeled MAb 9B9 in IPL or in rats in vivo demonstrated radioactivity only associated with a band above 140 kDa, close to molecular mass of IgG. Third, prolonged pulmonary retention of radiolabeled or b-MAb 9B9 after in vivo injection and in IPL, as well as modest release of cell-associated radiolabel in HUVEC, support the conclusion that endothelial cells internalize MAb 9B9 without marked degradation. Therefore, in terms of drug targeting, our results open a new avenue of utilization of MAb 9B9 as an affinity carrier for selective intracellular delivery of drugs and agents to the vascular endothelium.

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Targeting endothelium and its dynamic caveolae for tissue-specific transcytosis *in vivo*: A pathway to overcome cell barriers to drug and gene delivery

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Site-directed pharmacodelivery is a desirable but elusive goal. Endothelium and epithelium create formidable barriers to endogenous molecules as well as targeted therapies *in vivo*. Caveolae provide a possible, yet unproven, transcellular pathway to overcome such barriers. By using an antibody- and subfractionation-based strategy, we generated a monoclonal antibody specific for lung caveolae (TX3.833) that targets rat lungs after i.v. injection (up to 89% of dose in 30 min). Unlike control antibodies (nonbinding or to lipid rafts), TX3.833 targets lung caveolae that bud to form free vesicles for selective and quantal transendothelial transport to underlying tissue cells *in vivo*. Rapid sequential transcytosis can occur to the alveolar air space via epithelial caveolae. Conjugation to TX3.833 increases drug delivery to the lung up to 172-fold and achieves rapid, localized bioefficacy. We conclude that: (*i*) molecular heterogeneity of the endothelium and its caveolae permits vascular targeting to achieve theoretical expectations of tissue-specific delivery and bioefficacy; (*ii*) caveolae can mediate selective transcytosis *in vivo*; and (*iii*) targeting caveolae may provide a tissue-specific pathway for overcoming key cell barriers to many drug and gene therapies *in vivo*.

Molecular medicine has discovered many new therapeutic modalities by using state-of-the-art techniques in molecular biology. High through-put, *in vitro* assays that screen for pharmacological actions on the desired cell type are frequently used to design new drugs. Although such agents are certainly justified by their success *in vitro*, they frequently perform much less effectively *in vivo* where the agent must reach its target cells in a tissue in sufficient quantities to be potent while sparing bystander organs (1). Depending on the route of administration, the endothelium and/or epithelium form significant barriers that greatly limit the *in vivo* accessibility of many drugs, antibodies, and gene vectors to their intended target sites of pharmacological action, namely, the cells inside the tissue (1–4). For example, poor tissue penetration has hindered many monoclonal antibodies from reaching their cell-specific antigens to achieve effective tissue- or cell-directed pharmaco-delivery *in vivo* (1, 3–6). Moving the target from the tissue cell surface to the surface of vascular endothelium has theoretical advantages in tissue-specific delivery (7–10). This vascular targeting strategy still requires the identification of a tissue-specific target on endothelium, validation of expected delivery *in vivo* and, to be useful for many therapies, a means by which to enter and even cross the vascular wall for access to underlying tissue cells (7).

The microvascular endothelium in most organs acts as a significant barrier to the free passage of bloodborne molecules and cells to the underlying interstitium and tissue cells (8, 11). Specific transport mechanisms are expected to exist for the transendothelial transport of essential circulating blood macromolecules to the subendothelial space to meet the metabolic needs of the surrounding tissue cells (8). Continuous endothelium contains distinct flask-shaped invaginations in the plasma membrane called caveolae that are open to the luminal blood vessel space where circulating molecules may enter them (12–14). These caveolae may provide a trafficking pathway for macromolecules into and possibly across cells (7, 8, 11–14). Based on morphological studies showing few plasmalemmal vesicles existing free and unattached to other membranes inside the cell, some investigators have concluded that caveolae are not dynamic but rather static structures (15–18). Yet, caveolae can bud from the plasma membrane via a dynamin-mediated, GTP-dependent fission process (19, 20), and they contain key functional docking and fusion proteins (20–24). Whether caveolae can traffic their cargo across cells (transcytosis) remains unproven, primarily because comparative analysis has not been possible by using probes capable of targeting caveolae with high affinity and specificity *in vivo* vs. physically identical, nontargeting control probes. The utility of caveolae in overcoming cell barriers to facilitate efficient pharmacodelivery *in vivo* is unknown. The molecular composition of caveolae, including possible tissue-specific differences, is unknown.

Methods

Antibody Production. Monoclonal antibodies were generated by standard somatic cell hybridization using 100 μg of silica-coated luminal endothelial cell plasma membranes (P) as an immunogen and were screened by ELISA with P adsorbed onto 96-well trays.

***In Vivo* Biodistribution Studies.** IgG was purified by Protein G chromatography (Pierce) and radiolabeled with ^{125}I using Iodogen (25). The rat tail vein was injected with 10 μg of ^{125}I-IgG in 500 μl of rat serum albumin (10 mg/ml). After 30 min, the rats were anesthetized for thoracotomy, blood sampling by cardiac puncture, and organ removal. Tissues were weighed before counting γ radioactivity to determine antibody per g of tissue. In initial studies, ^{51}Cr-labeled red blood cells were injected to determine actual tissue uptake by subtracting tissue blood volumes. For TX3.833, this correction was negligible, and the practice was discontinued.

Antibody-Au Conjugates. A monodispersed solution of colloidal gold (average diameter = 6 nm) (EM Science) was adjusted to pH 9.2 with K_2CO_3 before adding purified IgG and stirring rapidly for 30 min. Polyethylene glycol (M_r 20,000) was added to a concentration of 0.5 mg/ml for the last 5 min. After centrifugation at 105,000 × *g* for 1 h at 4°C, the loose pellets were collected and resuspended in 5 mM phosphate before dialysis against 50 mM Tris during which NaCl was added slowly to a concentration of 150 mM. The conjugates were used within 48 h of preparation.

Abbreviations: P, silica-coated luminal endothelial cell plasma membranes; EM, electron microscopy; H, tissue homogenates; V, isolated caveolae; TTI, tissue targeting index; dgRA, deglycosylated Ricin A chain; 5'NT, 5'-nucleotidase.

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Tracking Antibody-Au Complexes Perfused *in Situ* and *in Vivo*. For the *in situ* experiments, the cranial lobe of the rat lung was perfused at 10 mmHg with PBS+ (PBS containing 3% BSA and 14 mM glucose) at 37°C followed by 6 ml of TX3.833-Au or control mouse IgG_1-Au in PBS+ (OD_{540} = 18). Pulmonary artery perfusion was limited to the cranial lobe by ligature exclusion of all other lobes of the lung. After 2, 5, 10, or 15 min, the lobe was flushed with 6 ml of PBS+ at 37°C before perfusion fixation with 2% (wt/vol) paraformaldehyde and 2.5% (wt/vol) glutaraldehyde in 0.1 M Na cacodylate (KII). The removed lobe was processed for Epon embedding and electron microscopy (EM) as described in (19). To track TX3.833-Au *in vivo*, a TX3.833-Au (15 nm) conjugate (500 μg Ab) was injected directly into rat tail veins. After 15 min, the rat was given intramuscular anesthesia and, as described above, the lungs were flushed with PBS+ followed by KII and processed for EM (19).

Morphometry. Randomly selected fields were examined and recorded at a final magnification of ×49,500. As described (13, 19), an image analyzer (Analytical Imaging Concepts, Roswell, GA) was used to determine the number of gold particles in caveolae per unit surface of linear plasma membrane (4 μm) and in the interstitium per unit surface area (μm^2) of each compartment. We also quantified the number of gold particles found in >150 well defined, apparently single caveolae (no other connecting caveolae or part of one visible in the section) that were clearly attached to the luminal or abluminal plasma membrane by their necks. To minimize variability from sectioning, only caveolae whose diameter exceeded 50 nm were used.

Immuno-Conjugations. Conjugation of ^{125}I to antibody was performed by using Iodogen (25). Deglycosylated Ricin A chain (dgRA) (Sigma) was radio-iodinated (25), reduced with 5 mM DTT and filtered through a Sephadex G25 (Amersham Pharmacia) column in phosphate-EDTA buffer (pH 7.5). N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (Pierce) were used to conjugate dgRA or ^{125}I-dgRA to antibody by disulfide linkage or thioether linkage, respectively (26). The antibody conjugates were protein G affinity-isolated.

Results

Novel Antibody Specific for Lung Caveolae. To identify tissue-specific vascular targets and to address whether caveolae can function in selective transport *in vivo*, we generated mouse monoclonal antibodies to rat lung P and their attached caveolae (22, 27). Our screening identified an IgG_1 monoclonal antibody, TX3.833, that seems to be both tissue- and caveolae-specific. Immunoblotting revealed that TX3.833 specifically recognizes a 90-kDa protein expressed in lung but not in other tissues and enriched in lung P relative to the tissue homogenates (H) (Fig. 1 *A* and *B*, and see Fig. 7, which is published as supporting information on the PNAS web site, www.pnas.org). Further subfractionation of lung P to isolate caveolae (V) showed that this antigen is concentrated in caveolae (Fig. 1*B*) similar to caveolin-1 but unlike the lipid raft marker, 5′nucleotidase (5′NT). Densitometry performed under equal protein loading conditions revealed >15-fold enrichments for this antigen both in P relative to H and in V relative to P (>225-fold in V relative to H; n = 3). TX3.833 antigen also was detected in caveolin-1-coated caveolae isolated from V by using caveolin-1 antibodies (28, and data not shown).

Antibody mapping of P reveals considerable molecular diversity of endothelia among organs with differential expression of transferrin receptor, thrombomodulin, 5′NT, and caveolin-1 (Fig. 1*A*). TX3.833 antigen is only detected in P from lung and not other organs. Rat tissue immunostaining confirmed TX3.833 reactivity in lung alveolar microvasculature but not in bronchial

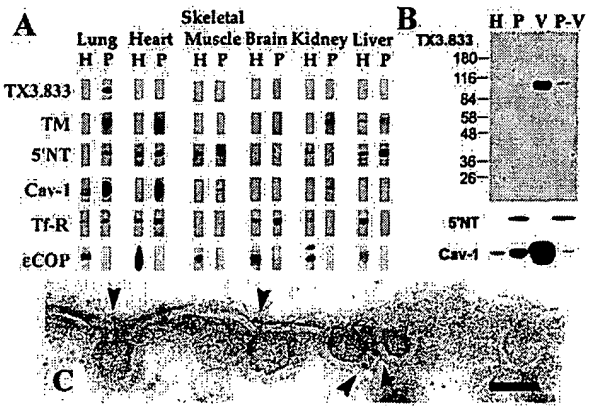

Fig. 1. Characterization of TX3.833. (*A*) Proteins (10 μg each lane) from the indicated H and P were subjected to Western analysis by using antibodies to the indicated proteins as in our past work (22, 27). (*B*) Proteins (5 μg) from rat lung H, P, V, and silica-coated plasma membranes stripped of caveolae (P-V) were immunoblotted by using TX3.833 and monoclonal antibodies to 5′NT and caveolin-1. (*C*) Ultra-thin cryo-sections of rat lung tissue were labeled with TX3.833 followed by reporter antibody conjugated to gold particles and were examined by EM. Arrowheads indicate endothelial caveolae. (Bar = 90 nm.)

epithelium, larger pulmonic vessels, or in any blood vessels of the heart, liver, brain, kidney, intestine, skeletal muscle, testes, spleen, skin, and adrenal (data not shown). More importantly, immunogold EM carried out on ultrathin frozen lung tissue sections showed that TX3.833 associated predominantly with the bulb and necks of the caveolae in microvascular endothelium (Fig. 1*C*, and Fig. 8, which is published as supporting information on the PNAS web site) and not clathrin-coated pits or epithelial cells (including their caveolae). Larger blood vessel endothelium of lung and controls using heart tissue or nonspecific mouse IgG_1 were negative (data not shown). Thus, TX3.833 specifically recognizes a 90-kDa antigen that is expressed selectively in caveolae of microvascular endothelium of lung but not other tissues.

Tissue Targeting *in Vivo*. To assess possible tissue-specific immunotargeting *in vivo*, we injected purified radio-iodinated TX3.833 or control IgG_1 into rat tail veins. Different biodistributions for each antibody were quite apparent 30 min after injection (Fig. 2 *A* and *B*). TX3.833 showed rapid and substantial lung uptake with very little detected in other organs (values less than or equal to control IgG) and very low blood levels (10-fold less than control). Up to 89% with a mean of 75 ± 6.4% of the injected dose of TX3.833 was targeted to the lungs in just 30 min. As in past reports (29), the control IgG remained in the blood with low tissue uptakes; the liver had the most, apparently because of IgG sequestration by F_c receptors. The lung tropism of TX3.833 was specific because unlabeled TX3.833, but not control IgG, inhibited lung accumulation by 89% and increased blood levels by >15-fold (Fig. 2*C*). Other antibodies generated in our screen recognized endothelial cell surface antigens in several tissues and accumulated *in vivo* in multiple tissues (data not shown). The uptake of TX3.833 significantly exceeds past reports describing various targeting probes (peptides and monoclonal antibodies), sometimes requiring up to 1 week to achieve a maximal tissue uptake of 0.2–4% of the injected dose (4, 6, 29–34). Even 24 h after injection, 46% of TX3.833 still remained in the lung.

Calculation of the tissue targeting index (TTI = antibody in tissue per g of tissue per antibody in blood per g of blood) and tissue selectivity index (TSI = TTI for targeting IgG per TTI for control IgG) confirmed lung targeting of TX3.833 with a mean

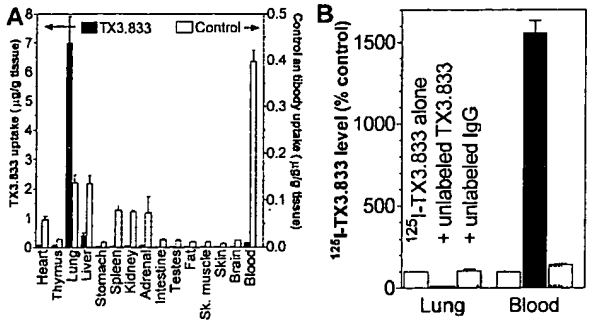

Fig. 2. In vivo biodistribution of TX3.833. (*A*) Blood levels and tissue uptake of ^{125}I-labeled TX3.833 and control IgG_1 (10 μg) were determined 30 min after i.v. injection. The mean value in μg IgG/g of tissue or blood is plotted with SD bars ($n \geq 3$). (*B*) Rats were injected with 1 mg of unlabeled TX3.833 or control IgG 30 min before ^{125}I-TX3.833 (10 μg) and assessed for radioactivity in the lung and blood, which is expressed as a percentage of signal without unlabeled antibody.

TTI of 56 and TSI of 150. The control IgG lacked targeting with TTI < 1 for all organs examined (maximum in liver of 0.36). Last, TX3.833 injections into the right vs. left ventricular chambers produced, even after 15 min, for both injections a TTI >10 for lung and <1 for other tissues. Thus, TX3.833 lung tropism depends not on first pass through the pulmonic circulation but rather on the antigen expression restricted to lung microvascular endothelium.

Transcytosis of Antibody Targeting Lung Caveolae *in Situ*. To assess possible targeting to and transport by caveolae of TX3.833, we perfused TX3.833 conjugated to colloidal gold particles (TX3.833-Au) through the rat pulmonary artery. EM and morphometric analysis (Fig. 3 and Fig. 4) revealed specific and rapid TX3.833-Au targeting to caveolae followed by transendothelial transport of the targeted cargo. Within 2–3 min, TX3.833-Au was found at the endothelial cell surface mostly bound to accessible luminal caveolae, either at their necks (on or near the diaphragm) or penetrating into the bulb of the caveolae (Fig. 3*A*, arrowheads). Very little-to-no TX3.833-Au was detected in caveolae located further inside the cell, attached to the abluminal cell surface, in clathrin-coated pits and vesicles or in the subendothelial space. After 5 min, penetration of TX3.833-Au into the caveolar system increased with many more caveolae containing gold particles including those connected at the luminal surface, further inside the cell, and at the abluminal surface (Fig. 3*B*, arrowheads). Occasionally, gold particles were seen inside (and not yet exiting) abluminal caveolae that opened onto the subendothelial space (data not shown). By 10 min, many gold particles were detected exiting the endothelium from the abluminal caveolae into the subendothelial space (Fig. 3 *C* and *D*, arrows). The amount of gold in the perivascular space was increasing but remained mostly in the proximity of the basal/abluminal endothelial cell plasma membrane (subendothelial space). Gold particles also were observed in racemose caveolar structures (Fig. 3*C*, thick arrow). There was little evidence of caveolae-mediated endocytosis to intracellular organelles such as endosomes (Fig. 3*E*). Although sometimes TX3.833-Au was seen accumulating at a luminal region near endothelial intercellular junction (Fig. 3*E*), little-to-no gold was found in the interstitium at the junctional exit (open arrow), consistent with a lack of transport through this pathway and the expected size-exclusion of the gold particles. After 15 min, TX3.833-Au still was being transported and existed in many luminal, abluminal, and apparently internalized caveolae (Fig. 3*F*, arrowheads). Gold particles continued to accumulate in the interstitium of the tissue (Fig. 3*F*, thick arrows).

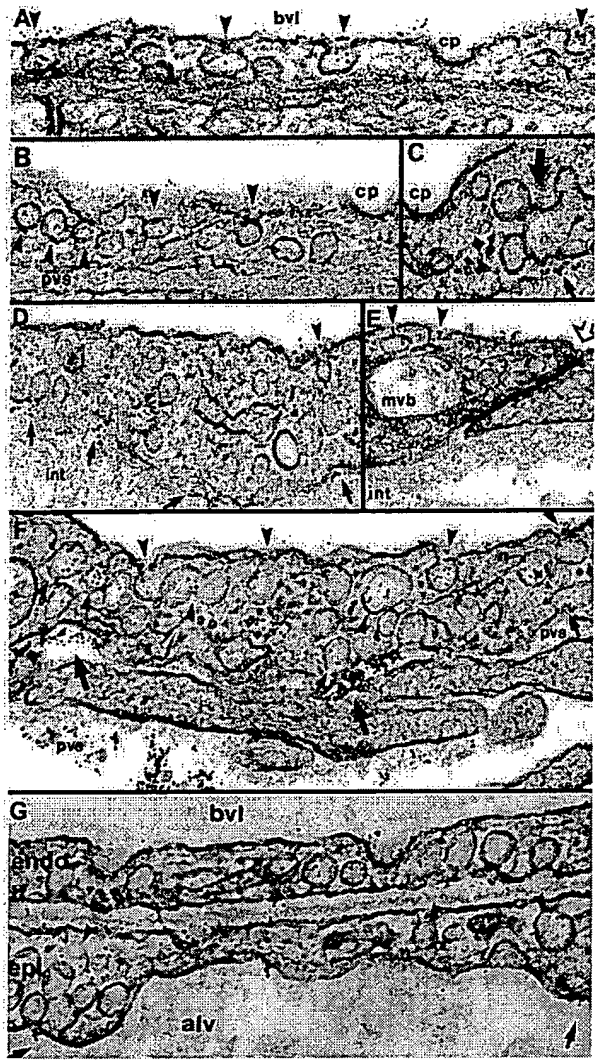

Fig. 3. Caveolar targeting and trafficking of TX3.833-Au *in situ*. The rat lung was perfused *in situ* with TX3.833-Au for 2.5 min (*A*), 5 min (*B*), 10 min (*C–E*), and 15 min (*F* and *G*), and processed for EM. Int, interstitial space; cp, coated pits; bvl, blood vessel lumen; pvs, perivascular space; mvb, multivesicular bodies; endo, endothelium; epi, epithelium; alv, alveolar space. [Bar = 90 nm (*A*), 128 nm (*B*), 116 nm (*C* and *D*), 124 nm (*E*), 90 nm (*F*), 85 nm (*G*).]

In several fortuitous cases, we caught the release of gold particles from the neck or introit of caveola (arrows, Fig. 3 *D* and *F*). The gold particles were seen as a cluster in the region immediately adjacent to the caveolar opening, apparently exiting as a bolus and just beginning to disperse freely into the underlying space. Sometimes one or two gold particles were still found just inside the neck of an otherwise empty abluminal caveola. No other gold particles were seen in the vicinity. Morphometric analysis showed that a similar number of gold particles entered luminal caveolae as exited abluminal caveolae. At 2.5 min, the labeled luminal caveolae clearly attached to the plasma membrane had a mean of 6.4 ± 0.5 particles per caveola with <25% of total luminal caveolae exhibiting no labeling or occasionally one or two gold particles. By 10 min, the mean number of gold

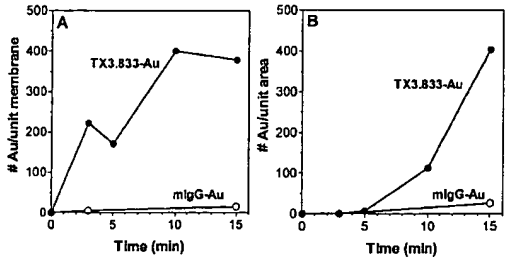

Fig. 4. Analysis of the binding and transport of TX3.833-Au vs. IgG-Au in rat lung. Comparative morphometric analysis was performed on the electron micrographs of rat lungs perfused with TX3.833-Au or mouse IgG-Au (see *Methods*) to quantify over time the number of gold particles (*A*) in lung microvascular endothelial caveolae per unit membrane and (*B*) within the interstitial space per unit area (μm^2).

particles inside labeled luminal and abluminal caveolae was 6.3 ± 0.3 and 7.1 ± 0.6, respectively, although, again, a subpopulation of caveolae (<25%) still had little to no label. Lastly, the mean number of gold particles exiting the neck of abluminal caveola was 6.7 ± 0.8 particles. This quantal uptake, transport, and release of six to seven gold particles per caveolae is consistent with discrete transcytosis of targeted molecular cargo by caveolae.

By comparing TX3.833-Au with physically identical probes having different binding specificity (i.e., all 150-kDa IgG_1 bound in the same way to the same size gold particles), we found that the ability of TX3.833 to specifically target the gold to caveolae mediates its transport across the lung microvascular endothelium. Even after 15 min, control mIgG-Au did not accumulate in caveolae or overcome the endothelial cell barrier (Fig. 9*A*, which is published as supporting information on the PNAS web site). Morphometric analysis showed that both entry to caveolae and interstitial accumulation were significantly more for TX3.833-Au than mIgG-Au (Fig. 4). As an additional control, we tested gold-conjugated antibody to 5′NT (5′NT-IgG-Au), a glycosylphosphatidylinositol-anchored endothelial cell-surface marker concentrated in lipid rafts but not caveolae (22). 5′NT-IgG-Au bound to the lung endothelial cell surface primarily in clusters on the plasmalemma proper that sometimes were at or near the caveolar diaphragm (Fig. 9*B*, arrowhead, which is published as supporting information on the PNAS web site). 5′NT-IgG-Au did not traverse the endothelium to accumulate in the interstitium even after 15 min. Targeting lipid rafts under equivalent conditions did not result in rapid transcytosis. Thus, the TX3.833-Au transported to in the interstitium depended on targeting caveolae.

Sequential Transcytosis. At 15 min with TX3.833-Au, we also found, where the attenuated endothelium was in close apposition to the alveolar epithelium, some of the gold particles accumulating in the subendothelial space percolated through the basement membrane to be taken up by epithelial caveolae for transport into and even across the cell (Fig. 3*G*, arrowhead). Gold particles were sometimes in endosomal-like structures and exiting caveolae opening into the air space (arrow). Although surprising because we detected little TX3.833 antigen in the epithelium by immunogold EM (Fig. 8), it is possible that where the interstitial space separating the endothelium from the epithelium is small enough to allow higher concentration of transcytosed gold particles, the epithelial caveolae could take up and transport the gold particles by fluid-phase mechanisms. Perhaps more likely, the TX3.833 antigen is expressed at lower levels in the epithelial caveolae. Thus, transcellular transport by caveolae occurs not only in endothelium but also in epithelium,

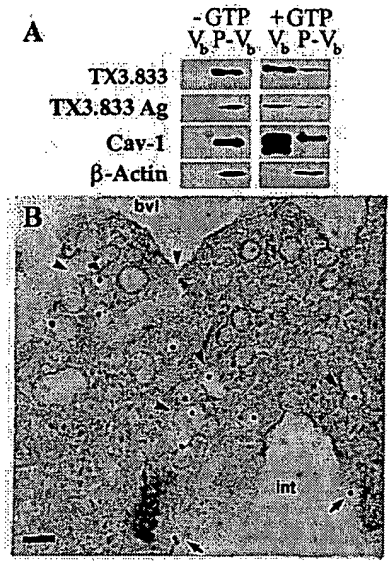

Fig. 5. TX3.833 targets dynamic caveolae *in vivo*. (*A*) Rat lungs were perfused for 5 min with 1 mg of biotinylated TX3.833 before flushing and silica perfusion for isolation of P that was subjected to the caveolae budding assay with or without GTP (20, 22). The budded caveolae (V_b), isolated by flotation away from the repelleted membrane (P-V_b), were immunoblotted with either streptavidin or the indicated antibodies. (*B*) After tail vein injection (15 min), TX3.833-Au is seen selectively in caveolae throughout the endothelial cell, consistent with transcytosis by the caveolae trafficking system. Arrowheads indicate a caveola or group of caveolae with gold particles inside. Arrows show gold particles exiting abluminal caveolae. Bvl, blood vessel lumen; int, interstitial space. (Bar = 91 nm.)

and this sequential transcytosis can be used to overcome rapidly both cell barriers.

TX3.833 Targets Dynamic Caveolae. Some caveolae may be static (15–18). To address whether TX3.833 targets dynamic caveolae capable of budding, we performed a reconstituted budding assay (19, 20) on P from lungs perfused with TX3.833. GTP induced plasmalemmal budding of caveolae that were collected as free floating vesicles containing the injected TX3.833 as well as caveolin-1 and TX3.833 antigen but not β-actin (Fig. 5*A*). This budding required GTP with active dynamin and was inhibited by nonhydrolyzable GTPγS and K44A mutant dynamin (19). Thus, TX3.833 can target dynamic caveolae that require GTP hydrolysis by dynamin for their fission from the plasma membrane to form free transport vesicles.

Tracking Caveolae Targeting and Transcytosis *in Vivo*. Proteins and possibly other elements in the circulating blood increase the restrictiveness of the endothelial cell glycocalyx and can prevent access to caveolae (12). To test the ability of TX3.833 to target lung caveolae *in vivo*, we injected TX3.833-Au into rat tail veins, and 15 min later, we processed the lung tissue for EM. TX3.833-Au could target the lung endothelial caveolae rather selectively (Fig. 5*B*). Gold label could be detected in luminal, abluminal, and apparently cytoplasmic caveolae. Transcytosis was observed with gold particles seen exiting abluminal caveolae to the underlying subendothelial space under normal physiological conditions *in vivo*. This visualization of caveolae targeting and transcytosis *in vivo* was consistent with the antibody-specific targeting of drug shown in Fig. 2 as well as our lung subfractionation analysis showing ^{125}I-TX3.833 (10 min after injection)

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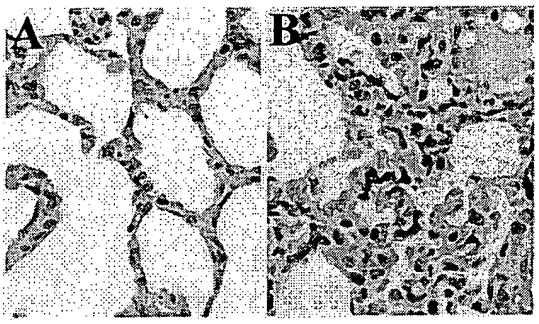

Fig. 6. Drug bio-efficacy studies *in vivo*. Hematoxylin and eosin staining of rat lungs 24 h after treatment with (*A*) control antibody-dgRA or (*B*) TX3.833-dgRA. Note the evident tissue damage with gross change in tissue morphology, infiltration of blood cells into tissue, edema, and septal thickening. (Bar = 1.3 μM.)

was enriched in V at levels 100-fold > ^{125}I-contol IgG (data not shown).

Lung-Specific Delivery and Bioefficacy of TX3.833-Drug Conjugates. An antibody targeting lung caveolae *in vivo* could be useful as a carrier to achieve tissue-specific drug delivery. To test TX3.833 as a targeting vector, we conjugated it to various drugs and examined *in vivo* delivery of the immunoconjugate relative to the native drug. All TX3.833-drug conjugates showed greatly increased lung targeting up to 172-fold greater than drug alone (see Table 1, which is published as supporting information on the PNAS web site). We also examined the bioefficacy of targeted drug by using dgRA immunotoxin, which is highly toxic in small amounts but requires internalization by cells for A chain release into the cytoplasm and subsequent cell death (35). At 6 h after i.v. injection, and even more so at 24 h after, the TX3.833-dgRA-treated rats showed acute respiratory distress and general malaise (very rapid, shallow breathing, remaining stationary, and focused on just breathing). Histological tissue examination revealed lung tissue disruption with edema, blood infiltration, and thickening of septa (Fig. 6) but no detectable damage to any other organs (e.g., see Fig. 10, which is published as supporting information on the PNAS web site). EM also revealed a loss of endothelial junctional integrity, marked membrane vesiculation of both endothelial and epithelial cells, and the presence of surfactant bodies in the alveolar spaces (Fig. 11, which is published as supporting information on the PNAS web site). The rats treated for 24 h with controls (equivalent levels of control IgG-dgRA, unconjugated TX3.833, native dgRA alone, or TX3.833 unconjugated but together with dgRA) appeared clinically and histologically normal (Fig. 10, which is published as supporting information on the PNAS web site). The damage to lung endothelial and epithelial cells is consistent with endothelial transcytosis of TX3.833-Au and uptake by underlying tissue cells. Thus, our cumulative data indicate that directing a drug to endothelial caveolae can provide tissue-specific targeting, transcytosis for access to cells inside the tissue, and localized bioefficacy *in vivo*.

Discussion

The concept of vascular targeting has evolved in the last 20 years from the failure of many directed therapies to reach their intended target tissue cells (4, 7–10). Targeting endothelium because of its inherent i.v. accessibility has potential, but so far requires key "proof of principle" *in vivo*. Although many attempts have been made to identify tissue-specific targets on vascular endothelium and to develop tissue-specific probes for vascular targeting (32, 33), directed delivery *in vivo* has not met theoretical expectations. A lung-targeting monoclonal antibody has been reported, but the antigen is thrombomodulin, which is expressed by many cells, including endothelia of several organs (ref. 32; Fig. 1*A*). Immunotargeting the pan-endothelial marker, PECAM, can improve delivery to the lung (5-fold over control IgG), but only when the antibody is biotinylated and complexed with streptavidin (36). Screening phage display libraries *in vivo* for tissue-homing peptides has provided modest increased tissue delivery (31, 33, 37) with <1% of the injected dose and relative targeting indices of 2- to 35-fold more than control phage and 3- to 80-fold more delivery than to the brain (note the well known blood–brain barrier has, as expected, the least nonspecific binding and uptake). By using this last criteria, TX3.833 targeting index is >1,000-fold more to lung than brain. TX3.833 as a probe has the specificity and affinity as well as the tissue- and cell-selectivity to validate, for the first time, the vascular targeting strategy by achieving theoretical expectations with high-level tissue targeting *in vivo*. More importantly, perhaps, it targets dynamic caveolae to overcome the endothelial cell barrier for access to underlying tissue cells.

Transport Pathway and Mechanism. Our cumulative *in vivo* and *in situ* data show that (*i*) caveolae can contain a tissue-specific endothelial protein, (*ii*) an antibody can selectively and rapidly target and enter caveolae of microvascular endothelium in a specific tissue, and (*iii*) targeting caveolae greatly increases the transendothelial transport and tissue accumulation over control antibodies (TTI $\geq$ 150). Little transport or tissue accumulation is observed with physically similar, isotype-matched control antibodies that differ from TX3.833 in their ability to recognize a specific caveolar antigen. Thus, it is the specific entry and binding within the caveolae and not just binding to the endothelial cell surface or another microdomain, such as lipid rafts, nor fluid-phase uptake by the caveolae that mediate the rapid and selective transendothelial transport of TX3.833-Au. The graded, time-dependent movement of the caveolae-targeting immunoprobes across the cell barrier cannot be explained by a nonspecific transport pathway (i.e., intercellular junctions) and back-filling of the abluminal aspect of a static, branched caveolar system, as logically proposed (18). Although it is now clear from appropriate controls that caveolae can mediate selective transendothelial transport, the exact mechanism for this transcytosis requires further elucidation.

Over the last 4 decades, two distinct but not mutually exclusive mechanisms for transendothelial transport by the caveolar system have been debated without resolution (8, 11, 15, 16, 18, 38–40): (*i*) active vesicular shuttling of dynamic caveolae that bud from the plasma membrane (20) or even from racemose caveolar structures (39) to form free caveolar transport vesicles carrying their molecular cargo across the cell to fuse with the abluminal membrane; and (*ii*) formation of continuous transendothelial channels through the linkage or temporary fusion of luminal and abluminal caveolae (either as a single caveola or as a group of racemose caveolar vesicles) effectively to form a patent large pore (40). The first mechanism moves a discrete packet of molecular cargo across the cell, whereas probes travel through and exit continuous transendothelial channels as a steady stream of molecules (40). TX3.833 moves across the endothelium not as a continuous stream but rather in discrete packets (six to seven gold particles inside each luminal and abluminal caveola as well as exiting from a then-empty abluminal caveola). The simplest trafficking mechanism consistent with the data has caveolae moving their units of molecular cargo only a short distance across the cell as dynamic, discrete vesicular carriers. Caveolae, like various known trafficking vesicles, have their own molecular machinery to mediate vesicle budding, docking, and fusion, including dynamin, vesicle-associated membrane protein-2, *N*-ethylmaleimide-sensitive factor (NSF), and

soluble NSF attachment proteins (19–24). Here, we show TX3.833 targeting of dynamic caveolae requiring dynamin and GTP hydrolysis to bud from the plasma membrane to form free transport vesicles (19, 20) still carrying the TX3.833 cargo (Fig. 5). These data and the quantal transport of TX3.833-Au support a transcytosis model, in which caveolae bud to transport their targeted cargo into and across the cell.

Large clusters of interconnected caveolae forming cavernous, racemose structures, possibly related to vesiculo-vacuolar organelles (41), may transport a minor portion of TX3.833. Dynamin located at caveolar necks (19) may mediate the budding of individual caveolae or even the whole racemose structure, which may explain the 3- to 5-fold greater amounts of gold probes exiting racemose caveolar structures than any single caveola (e.g., see Fig. 3*C* showing a caveolae racemose structure releasing 25 gold particles). Outside the perinuclear region, endothelial cells, especially in capillaries, are very attenuated, with distances between luminal and abluminal plasma membranes less than or equal to two caveolae diameters (0.1 μm). Most transvascular exchange (11) occurs across such regions with high surface area to volume ratios and little room for racemose caveolar structures. We find the transendothelial transport of TX3.833 occurs mostly in these attenuated regions with the single caveolae carrying their targeted cargo a very short distance. This attenuation may not only speed transendothelial transport into the tissue but also benefit recycling of caveolae back to the luminal surface.

Application for Site-Directed Pharmacodelivery. A selective caveolae targeting strategy may be useful for directed therapeutic delivery for the treatment of many diseases. By targeting caveolae at the luminal surface of endothelium in a single tissue, where the caveolae can transport the antibody from the blood directly into the target tissue, we find that antibodies indeed can provide site-directed delivery *in vivo* with tissue accumulations reaching as high as 89% in just 30 min. Much of this lung-specific accumulation is antibody that has crossed the lung endothelial cell barrier by caveolae-targeted transport to become available for uptake by underlying tissue cells. Here, we have visualized this process by EM to show TX3.833 directing gold particles to the lung caveolae for rapid transendothelial transport from the circulating blood into the tissue. Like many drugs and gene vectors, the gold particles, alone or conjugated to control antibodies, do not readily cross the endothelial cell barrier. When conjugated to potential drugs or toxins, drug delivery by TX3.833 was selective to the lung and enhanced by up to 172-fold. TX3.833-dgRA conjugates selectively damaged the lung, primarily through the destruction of the alveolar endothelial and epithelial cells. Thus, an antibody targeting caveolae can be a carrier to provide tissue-specific pharmacodelivery and bioefficacy through overcoming the endothelial cell barrier that normally restricts delivery to the underlying cells of the tissue.

In our quest to study the basic transport function of caveolae in endothelium, we have shown that caveolae indeed can transcytose select cargo in endothelium as well as epithelium. We have produced and characterized a lung-, microvessel-, and caveolae-specific monoclonal antibody that targets the lung *in vivo*. After intravascular administration, TX3.833 rapidly targets dynamic caveolae of the lung microvascular endothelium and is rapidly transported across the cell for release to the interstitial space where it can be taken up by the caveolae of underlying epithelial cells and transported to the alveolar space. As such, caveolae may provide a selective and useful vesicular trafficking pathway not only to endocytose or transcytose select endogenous molecules but also to overcome the once seemingly insurmountable cell barriers to effective site-directed therapeutic delivery *in vivo*. Moreover, proteomic analysis of endothelial cell plasma membranes and their caveolae reveals several tissue-specific proteins that are differentially expressed in normal organs and in various solid tumors (our unpublished data). Thus, a strategy of targeting caveolae of endothelium and epithelium offers exciting possibilities for achieving site-directed drug and gene therapy of various diseases *in vivo*.

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Review article

Immunotargeting of drugs to the pulmonary vascular endothelium as a therapeutic strategy

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1. Introduction

Drug targeting has been created as a speculative concept at the very beginning of the century by one of the founders of immunology, Paul Erlich. Since he discovered antibodies and their role in the humoral immunity, Erlich proposed that antibodies may serve as 'magic bullets' delivering therapeutic agents to the target cells. That was not earlier than two decades ago, however, when invention of monoclonal antibodies provided a scientific background for experimental exploration of Erlich's brilliant idea. Thus the modern concept of drug targeting evolved in the late seventies and emerged nowadays as a novel field of experimental biomedicine.

The ultimate goal of drug targeting is a strategy for specific, effective and safe therapeutic interventions localized at the target site. Unfortunately, most of known drugs do not accumulate in their targets. This restricts effectiveness and safety of therapy. Administration of high doses in order to compensate for drug dilution, inactivation and elimination causes side effects, immunological reactions and imposes inconvenience in treatment (prolonged infusions, e.g.). Specific delivery of drugs to the target should minimize or reduce these obstacles. In addition, precise intracellular addressing is required for effective action of certain drugs. Therefore, drug targeting should provide both target recognition and proper intracellular trafficking of a drug.

The paradigm of the strategy is that conjugation of a drug with a carrier molecule recognizing specific determinants presented on surface of the target cells will provide selective delivery of a drug to these cells. A carrier molecule can be chemically conjugated to either a drug or a drug vehicle, such as liposome, red blood cell, protein or polymer carrier. Numerous methods for chemical conjugation, as well as gene engineering methods for creation of chimeras combining antigen-binding site of a carrier antibody and therapeutic enzyme have been developed. Analysis of the literature on the drug targeting and methods for conjugation lies beyond the scope of this paper; readers may address directly to numerous reviews and handbooks on the subject (see, for example [3,57,61,88,135,139,159,171,174]).

Initially, most of studies in the field were focused in oncology, because targeting of radiolabels would be helpful for detection and visualization of tumors, whereas targeting of antibiotics or toxins (immunotoxins) would provide tumor-specific chemotherapy. Nowadays, numerous laboratories develop systems for targeting of antibiotics, radiolabels, toxins, antioxidants, fibrinolytics, hormones, antisense oligonucleotides, genes and other agents to hepatocytes, Kupffer cells, epithelial cells, pneumocytes, antigen-presenting cells, macrophages, vascular cells, blood cells, fibrin clots, myocardium, brain, joints, tumors, and other targets.

Endothelial cells also represent an important target for therapeutic or experimental interventions. Endothelium is an interface between blood and tissues, transducing chemical and mechanical signals and performing nutrition function. Endothelial cells regulate blood coagulation and fibrinolysis, as well as control activity of blood and vascular cells via production and degradation of vasoactive compounds and intercellular messen-

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gers such as angiotensins, substance P, bradykinin, nitric oxide, oxidants, cytokines and growth factors. Endothelium, however, is vulnerable to action of damaging agents such as proteases, oxidized LDL, vasoactive compounds (histamine, thrombin, angiotensins, etc.), as well as oxidants and agents generated in course of activation of leukocytes, complement, coagulation and fibrinolysis (for reviews see [64,167]). Therefore, development of the strategy for specific and effective treatment for endothelial cells is an important biomedical goal.

Various ligands (hormones, lectines, growth factors, etc.) may be useful as carriers for targeting of drugs to determinants presented on the surface of either normal or pathologically altered endothelial cells [73]. All these determinants, however, may serve as antigens, i.e. specific targets for antibodies utilized as drug carriers. Accordingly, major efforts in the field are focused on the exploration of antibodies recognizing endothelial surface antigens (i.e. immunotargeting). As we will see below, some antibodies may preferentially recognize endothelial cells localized in certain organs or vascular areas. For example, immunotargeting to the endothelial cells localized in the tumor vasculature may provide a novel avenue for site-specific treatment of solid tumors [20,69,81]. For the sake of focus, this paper will pay specific attention to immunotargeting to the pulmonary endothelium, which represents an important target for site-specific therapy. The purpose of this review is to analyze previous achievements, present status, potential problems and perspectives of immunotargeting to the normal or pathologically altered endothelial cells localized in the pulmonary vasculature.

2. Pulmonary endothelium as a target for site-specific therapy

Pulmonary endothelium represents extremely large specific domain in the vasculature and executes very important functions, both local and systemic. In addition to general endothelial functions listed above, pulmonary endothelial cells (EC) control vascular permeability in the lungs, regulate pulmonary and systemic vascular tone, manage interaction of blood cells with pulmonary tissue, maintain balance between blood coagulation and fibrinolysis in the lung, and contribute to immunological reactions in the lung tissue (for overview of the pulmonary endothelium, see a book edited by Ryan [142]). Pulmonary endothelium, however, is anatomically predisposed to injury induced by or associated with inhalation of oxygen metabolites and microparticles, sequestration of activated leukocytes in the pulmonary capillaries and deposition of blood clots and fibrin [17,52,63,74,111,150]. Pulmonary EC are sensitive to injury caused by systemic administration of certain drugs (e.g. bleomycin) and often suffer secondary injury in response to remote extrapulmonary disease condition, such as ischemia reperfusion of mesentery, liver or limbs [24,63]. Injury to the pulmonary endothelium plays an important role in pathogenesis of a wide variety of diseases and syndromes including pulmonary edema, adult respiratory distress syndrome (ARDS), sepsis, lung transplant rejection, lung radiation injury, pneumonia, dissemination of malignant cells in the lung, oxidative lung injury, lung ischemia reperfusion, pulmonary hypertension, lung inflammation and fibrosis [17,42,50,63,93]. Ideal treatment for the most of the listed disease conditions must provide specific therapeutic intervention in the pulmonary endothelium.

There are several approaches to deliver a drug to the lung. The lung is a unique organ in terms of possibility of local (i.e. intratracheal) administration of a drug. Intratracheal administration provides high concentration of a drug in the lung tissue and its prolonged contact with alveolar cells and, therefore, is useful for local application of enzymatic or gene therapy [11,129,136]. Endothelial cells, however, are inaccessible from the alveolar space and, therefore, receive a only minor (perhaps insufficient) portion of a drug. This point is illustrated by the recent work of Prayssak and co-authors cited above [136], where intratracheal transfer of genes encoding antioxidant enzymes afforded an effective protection against oxidative lung injury caused by hyperoxia-induced (where alveolocytes are probably the primary target), but not by ischemia reperfusion (where endothelial cells are the primary target [4]).

Although endothelial cells are not accessible from the airways, they are easily accessible from the circulation. Thus, intravenous route of administration seems to be ideal for drug delivery to EC. Conjugation of a drug with microspheres or encapsulation in a large liposomal aggregates provides drug accumulation in the lung vasculature due to mechanical retention in the pre-capillary vascular area. This approach is clinically applicable for visualization of the pulmonary vascular bed using radiolabel covalently bound to microspheres. However, elimination of the released drugs by the bloodstream will profoundly compromise the transfer of a drug from the carrier microspheres or liposomes to endothelial cells.

In order to avoid this obstacle, a drug may be conjugated with antibodies or antibody fragments recognizing the surface endothelial antigens. Since endothelium is directly accessible to the circulating blood, such conjugates should easily bind to the target. Thus, immunotargeting may be useful for the site-specific therapy of endothelial cells. Pulmonary endothelium is specifically attractive site for immunotargeting. First, pulmonary vasculature represents extremely large surface area containing about 30% of the total amount of

endothelial cells in the body. Second, pulmonary endothelium is the first large vascular target after intravenous injection of a drug and lung receives a whole cardiac blood output. Therefore, even carriers which do not discriminate between pulmonary and systemic endothelial cells may accumulate in the lung. Third, pulmonary endothelium is enriched in certain surface antigens (see below). This may provide an additional factor for tissue selectivity of the targeting.

3. Endothelial antigens as potential targets for drug delivery

The minimal requirement for immunotargeting to endothelium is that an antigen must present on the luminal surface of the endothelial cells. Ideally, it should not be present in the blood, since circulating antigen will compete with endothelial target for binding of the antibody-conjugated drug. For example, although fibronectin is a good model antigen for immunotargeting to endothelium in cell culture [116], its high concentration in the plasma preclude effective binding of fibronectin antibodies to endothelium in vivo. On the other hand, the presence of an antigen on other cells (smooth muscle cells, fibroblasts, etc.) in addition to endothelium does not preclude the endothelial targeting, because these other cells are not accessible to blood. In other words, even if non-endothelial cells buried in the tissues contain an antigen presented on the endothelial surface, the major portion of the intravascularly injected antibody will bind to endothelial cells. This point is illustrated by biodistribution of monoclonal antibody (mAb) against angiotensin-converting enzyme, ACE (described in details below). This mAb, traced by radiolabel tag, accumulates selectively in the lungs after systemic injection in animals [37,38] despite the fact that renal, intestinal and testicular content of ACE is higher than that in the lung [38]. This result may be explained by the fact that the major fraction of ACE in the lung is associated with vascular endothelium and, therefore, is easily accessible to blood. In contrast, major fraction of ACE in the kidney, gut and gonads is localized in epithelial cells [46] and, therefore, ACE in these organs is poorly accessible to the circulating antibody.

There are several issues which are important in estimation of applicability of an antigen as a target for drug delivery, such as:

1. Antigen localization and surface density in endothelium. Does it provide binding of substantial amount of antibody-conjugated drug?
2. Antigen function(s) and effect(s) of antibody binding to antigen. Does antibody or conjugate inhibit antigen function and what would be consequence(s) of this effect?
3. Effect(s) of pathological factors on the level of an antigen in endothelium. May certain pathological settings cause suppression or facilitation of immunotargeting?
4. Internalization, intracellular trafficking and degradation of an antigen or antigen-antibody complex. What is cellular destination of delivered drug? Is delivered drug stable and active?
5. An antigen distribution in the vasculature. Is it uniform in all vessels or endothelial cells in specific vascular areas (e.g. pulmonary endothelium) express more copies of antigen per cell and thus would be preferential targets?

Below we describe several endothelial antigens potentially useful as targets and analyze major advantages disadvantages of known carrier antibodies (see Table 1).

3.1. Angiotensin-converting enzyme (ACE)

Historically, ACE was one of the first antigens explored as an endothelial target. ACE is a carboxypeptidase constitutively localized on the luminal surface of endothelial cells [21]. ACE converts angiotensin I to angiotensin II, as well as inactivates bradykinin and some other vasoactive peptides [46]. In cell culture, ACE is diffusely distributed on the apical surface of endothelial cells [141]. Endothelial ACE is a 170-kD transmembrane glycoprotein with a short hydrophobic domain near the C-terminus that anchors the enzyme in the plasma membrane, whereas a large portion of endothelial ACE is exposed to the bloodstream from the luminal surface of the cell [29]. There is about $1-3 \times 10^5$ binding sites for anti-ACE antibodies on the luminal surface of a single endothelial cell [125]. The blood level of ACE is low as compared with its concentration in the kidney, gut and lung [31,46]. Ionophores and hypoxia stimulate ACE synthesis in endothelial cell cultures [32,83], thus implying that ACE-directed targeting to endothelium may be facilitated at certain pathological settings. This issue, however, is controversial and will be considered below. Recent study documented that pulmonary vascular endothelium is significantly enriched in ACE: at immunomorphological examination, 100% of the pulmonary capillaries are ACE-positive, vs about 20% of ACE-positive capillaries in other tissues [51]. This important observation helps to explain very high selectivity of the pulmonary targeting of ACE antibody described below.

Results of an early exploration of ACE antibodies as carriers for the vascular targeting were rather disappointing, since neither polyclonal nor monoclonal antibodies accumulated in the lung or other highly vascularized tissues after intravascular administration in animals [99,102]. The reasons for those negative results remain unclear and could include low affinity of

Table 1
Major properties of the targeting systems described in the present paper

Target antigen	Antigen characteristics	Potential advantages of the targeting system	Potential disadvantages of the targeting system
Angiotensin-converting enzyme, ACE	TMGP constitutively localized on the apical surface of EC. Pulmonary EC are enriched in ACE. EC internalize anti-ACE. ACE converts AngI to AngII and inactivates bradykinin.	1. Tissue-selective and effective targeting to the normal pulmonary EC.	1. Cytokines and ROS suppress the targeting.
		2. Intracellular delivery of drugs or genes. Modest rate of the intracellular degradation.	2. Inhibition of ACE may lead to potentially harmful side effects in the hypotensive patients.
		3. Suppression of ACE activity by anti-ACE may be beneficial in hypertensive patients.	
		4. A single iv injection of anti-ACE mAb is well tolerated by animals and patients.	
		5. Possibility for preventive administration.	
		6. Well documented targeting of various drugs to the pulmonary endothelium in animals.	
		7. Documented lung gamma-imaging with ^{111}In-anti-ACE in humans.	
Thrombomodulin, TM	TMGP constitutively localized on the apical surface of EC. Pulmonary EC are enriched in TM. EC internalize TM and its ligands. TM binds thrombin and converts it to an anticoagulant enzyme.	1. Tissue-selective and very effective targeting to the normal pulmonary EC.	1. Cytokines and ROS suppress the targeting.
		2. Intracellular delivery of drugs or genes.	2. Inhibition of TM may cause hypercoagulation and harmful side effect of blood clotting.
		3. Well documented targeting of various drugs and DNA to the pulmonary endothelium in animals.	3. Fast intracellular degradation of a drug.
Intercellular adhesion molecule-1, ICAM-1	TMGP constitutively localized on the apical surface of EC. Cytokines and ROS stimulate ICAM expression. EC poorly internalize ICAM. Serves as a counterpart for CAM on the blood cells and contributes to leukocytes binding to EC.	1. Targeting to normal or activated EC in the lung and other organs (liver, mesentery, stomach, etc.).	1. Relatively poor tissue selectivity of the pulmonary targeting.
		2. Targeting is stimulated by cytokines and ROS.	2. Lack of the intracellular delivery.
		3. EC surface targeting.	3. Lack of the published studies on the drug targeting in vivo.
		4. Possibility for extrapulmonary targeting.	
		5. Laboratory animals tolerate well a large dose of anti-ICAM.	
		6. Secondary therapeutic benefit of the inhibition of leukocyte-mediated injury.	

Table 1 (*Continued*)

Target antigen	Antigen characteristics	Potential advantages of the targeting system	Potential disadvantages of the targeting system
Platelet-Endothelial Adhesion Molecule-1, PECAM-1	TMGP constitutively localized on EC in the intercellular borders. Very high binding capacity for anti-PECAM on EC. PECAM contributes to leukocytes transmigration through EC and EC signal transduction.	1. Theoretically, high delivery capacity of the targeting to the vascularized tissues.	All as ICAM-1.
		2. Laboratory animals tolerate well administration of a large dose of anti-PECAM.	
		3. Secondary therapeutic benefit of the inhibition of leukocyte-mediated injury.	
E-selectin	TMGP expressed on EC activated with cytokines or ROS. EC internalize anti-E-selectins. Serves as a counterpart for CAM on the blood cells and contributes to leukocytes binding to EC.	1. Specific targeting to the inflammation-engaged (activated) EC.	1. Tissue selectivity of the pulmonary targeting is relatively poor.
		2. Prolonged recognition of the activated EC.	2. Effectiveness of the targeting is relatively low.
		3. Intracellular delivery of drugs.	3. Delayed (hours after an insult) recognition of the activated EC.
		4. Possibility for extrapulmonary vascular targeting.	4. Lack of the published studies on the drug targeting in vivo.
		5. Potential benefit of inhibition leukocyte-mediated injury.	
		6. Laboratory animals tolerate well a large dose of anti-E-selectin.	
		7. Published studies on radioimaging in animals and humans.	
P-selectin	Glycoprotein stored intracellularly in EC. Cytokines and ROS cause its rapid mobilization to EC plasma membrane. Contributes to adhesion of blood cells to activated EC.	1. Specific targeting to the activated EC.	1. Short-time (few hours) window for the targeting.
		2. Very rapid targeting to the activated EC.	2. Lack of the published studies on the targeting in vivo.
		3. Potential benefit of inhibition leukocyte-mediated injury.	3. The rate of internalization and cellular addressing are unknown.
		4. Laboratory animals tolerate well a large dose of anti-P-selectin.	4. A large pool of P-selectin may be exposed to the blood by activated platelets.

Please note that in some cases, advantage and disadvantage of a targeting system have a 'mirror-like' character and represent the same features of an antigen or a carrier antibody. Thus, inhibition of ACE by anti-ACE may be either beneficial or harmful at different disease conditions. Antibodies to cell adhesion molecules (anti-CAM) display relatively poor tissue selectivity of the pulmonary targeting (as compared with anti-ACE or anti-TM), but anti-CAM could be better carriers for targeting to the extrapulmonary EC. Lack of internalization (e.g. anti-ICAM), restricts intracellular targeting, but may provide the surface targeting required for such drugs as anticoagulants. Constitutive expression of an antigen (ACE, TM) precludes specific recognition of the activated EC, but allows pre-treatment regimens of drug targeting to EC (e.g. prevention of oxygen toxicity in patients with mechanical ventilation). Anti-E-selectin provides relatively delayed recognition of the activated EC, but targeting may have a prolonged therapeutic window; in opposite, P-selectin targeting will be limited by several hours after an insult, but anti-P-selectin provides very rapid recognition of the activated EC.

Abbreviations used in the Table: ACE, angiotensin-converting enzyme; TM, thrombomodulin; TMGP, transmembrane glycoprotein; CAM, cell adhesion molecules; EC, endothelial cells; mAb, monoclonal antibody; angI and angII, angiotensin I and II; ROS, reactive oxygen species.

antibodies used, lack of cross-reactivity with animal ACE or and poor accessibility of specific ACE epitopes recognized by those antibodies. Nevertheless, a decade ago, Dr Sergei Danilov at the National Cardiology Research Center in Moscow produced a new monoclonal antibody to ACE, mAb 9B9 [33]. This anti-ACE mAb 9B9, murine IgG1, cross-reacts with human, monkey, rat, hamster and cat ACE and reveals vascular endothelium in tissue sections obtained from these animal species [33,34]. This antibody does not inhibit enzymatic activity of purified ACE in solution, does not fix complement and induces no injury to endothelium either in cell culture of after in vivo administration at doses 100 mg·kg [37,38]. Radiolabeled mAb 9B9 accumulates selectively in the lungs after intravenous, intraarterial and intraperitoneal injections in rat, cats and hamsters [37,38]. Tissue-selective pulmonary accumulation of anti-ACE mAb 9B9 radiolabeled with either ^{99m}Tc or ^{111}In has been documented in monkeys and healthy human volunteers using gamma-immunoscintigraphy [35,121]. Endothelial cells in culture, in perfused rat lungs and rat lungs in vivo internalize mAb 9B9 [125]. Noteworthy, internalized mAb 9B9 undergoes relatively modest intracellular degradation and can be detected in form of an intact IgG-size radiolabeled protein in the lung homogenates even several days after intravenous injection [125]. Biotinylated mAb 9B9 retains complete functional activity in vitro and in vivo and affords highly effective pulmonary accumulation of ^{125}I-streptavidin, thus providing methodological background for universal system for immunotargeting of various biotinylated agents to the lung [120,122]. All these results strongly suggest that anti-ACE mAb 9B9 is an excellent carrier for the intracellular delivery of drugs (and possibly, genes) to the pulmonary endothelium.

Although extremely attractive as a carrier for the tissue-selective pulmonary immunotargeting, antibody to ACE is not an ideal choice in certain cases. First, cytokines, oxidants and other inflammatory mediators down-regulate ACE expression in endothelial cells and stimulate ACE shedding from endothelial plasma membrane [8,132,172]. Therefore, inflammation or oxidative stress may inhibit immunotargeting to ACE. Indeed, pulmonary vascular injury caused by administration of endotoxin, PMA, PAF or α-napthylthiourea in rats leads to marked suppression of the pulmonary targeting of ^{125}I-labeled anti-ACE mAb 9B9 [7,119,121]. Pulmonary sequestration of tumor cells injected intravenously in hamsters also reduced pulmonary targeting of mAb 9B9, probably, due to masking of the endothelial surface by adherent tumor cells [36]. Thus, immunotargeting of mAb 9B9 could be compromised at certain pathological conditions (although this can be useful for diagnostics by gamma-immunoscintigraphy, see below). Second, although mAb 9B9 does not inhibit ACE enzymatic activity directly [39], it accelerates ACE shedding from the endothelial plasma membrane [38] and via this mechanism reduces ACE activity in the lung [37,38]. ACE inhibitors are used clinically to down-regulate blood pressure in hypertensive patients and to protect vascular wall against restenosis [60]. Therefore, inhibiting effect of anti-ACE may provide a secondary benefit for therapy in certain cases, e.g. treatment of hypertensive patients. However, in patients with an acute hypotensic conditions (e.g. patients with trauma developing ARDS), ACE inhibition and subsequent vasorelaxation may be rather dangerous because of the high probability of vascular collapse. Therefore, application of anti-ACE in hypotensic patients is questionable. With all reservations, however, anti-ACE mAb 9B9 is one of the most selective and best characterized carriers for immunotargeting to the pulmonary endothelium, well tolerated by laboratory animals and patients.

3.2. Thrombomodulin (TM)

Thrombomodulin is a transmembrane endothelial glycoprotein (≈ 140 kD) with a large portion (N-domain) exposed to the bloodstream [48]. Similarly to ACE, TM is diffusely distributed on the apical surface of endothelial cells [68]. Binding of thrombin to TM leads to conversion of thrombin enzymatic activity: TM-bound thrombin activates anti-coagulant protein C, instead of cleavage of fibrinogen and thus TM controls coagulation cascade in the blood [48]. From the targeting standpoint, TM is similar to ACE in the sense that:

1. It is accessible to the bloodstream;
2. It is normal endothelial antigen; and
3. Pulmonary microvasculature is enriched in TM [55].

In the late eighties, Kennel and co-workers [78], have produced a set of antibodies reacting with murine thrombomodulin, anti-TM [55,145]. They have studied biodistribution of radiolabeled anti-TM mAb after iv injection in mice and have reported that it specifically accumulates in the murine lungs [79]. Effectiveness of the pulmonary accumulation was extremely high and reached 276% of injected dose of ^{125}I-anti-TM per gram [80]; this indicates that about 50% of injected dose accumulates in the target organ (average murine lung weight is 0.2 g). Pulmonary uptake of ^{111}In-anti-TM mAb in mice has been visualized by gamma-immunoscintigraphy [79] and by microradioautography of the lung tissue sections [80]. These results implied that anti-TM may be useful for drug targeting to the pulmonary endothelium.

Thrombomodulin undergoes constitutive endocytosis in endothelial cells [26]. In accordance with this fact, several groups have reported that endothelial cells rapidly internalize antibodies to TM [18,26,68]. How-

ever, mechanism(s) of internalization of TM and its ligands is not completely understood. Deletion of intracellular domain of TM does not eliminate its constitutive endocytosis [27]. Several lines of evidence imply that TM internalization is controlled by its extracellular portion. Certain ligands (thrombin, anti-TM) have been reported to stimulate TM endocytosis [101], whereas another ligand, protein C, has been shown to suppress endocytosis [95]. Deletion of N-terminal lectin-like portion of the TM extracellular domain inhibits its endocytosis [28]. Recent results show that endothelial cells poorly internalize a monoclonal antibody recognizing specific chondroitin sulfate-dependent epitope of thrombomodulin [126]. Therefore, speculatively, TM antibodies may provide either intracellular or surface delivery of drugs, depending on the properties of a carrier antibody.

Similar to ACE in many aspects, TM also is not an ideal target. First, cytokines, oxidants and oxidized lipoproteins down-regulate surface density of TM on the endothelial cells [71,89,101]. Therefore, inflammation will suppress immunotargeting to TM. Second, most of internalizable TM ligands (thrombin and anti-TM antibodies) undergo rapid intracellular degradation [18,94]. Thus TM-mediated targeting seems to be directed to lysosomal compartment, which may be inappropriate for certain drugs. Third, either antibody-induced internalization or antibody-mediated steric masking of TM on the endothelial plasma membrane may reduce its ability to bind and convert thrombin. Therefore, immunotargeting to TM may cause pro-thrombotic events due to suppression of an anticoagulant activity of endothelium. Furthermore, administration of TM antibody at dose 0.1 mg led to significant enhancement of subsequent colonization of injected tumor cells in the pulmonary vasculature, perhaps due to minor endothelial injury [78]. Therefore, the safety is of great concern in terms of utilization of TM antibodies for the drug targeting.

3.3. Constitutive surface adhesion molecules, ICAM-1 and PECAM-1

Endothelial cells constitutively express surface adhesion molecule ICAM-1 (intercellular adhesion molecule-1, CD54). ICAM-1 is a transmembrane glycoprotein diffusely distributed on the apical surface of endothelial cells [5], easily accessible from the bloodstream and participating in adhesion of leukocytes to endothelium [14,86,154]. Antibodies against ICAM-1 provide intense immunostaining of the vascular and epithelial cells in the normal lung tissue, although endothelial cells in large veins in the normal rat lungs display relatively poor immunostaining [9,19]. Importantly, proinflammatory agents TNFα, IL-1 and IFγ, as well as oxidants, stimulate ICAM-1 synthesis in endothelial cells [2,14,16,134,144,154]. Inflammation, therefore, causes up-regulation of ICAM-1 in the lung and other organs [12,19]. Therefore, ICAM-1-mediated immunotargeting may be facilitated in the vascular area(s) engaged in inflammation.

Tamatani and Miyasaka [156] described a monoclonal antibody reacting with rat ICAM-1, mAb 1A29. Since than, numerous laboratories studied biodistribution of ^{125}I-mAb 1A29 in normal rats and animals challenged with pro-inflammatory agents, such as endotoxin and TNFα. In accordance with high level of constitutive endothelial expression of ICAM-1 and its high accessibility to blood, ^{125}I-mAb 1A29 accumulates in the lungs of control rats [12,108,110,131,166]. In contrast with ACE and TM antibodies, which display tissue-selective pulmonary accumulation, ^{125}I-mAb 1A29 also accumulates in other highly vascularized tissues, such as mesentery, liver and spleen [131]. Experiments in mice utilizing antibody recognizing murine ICAM-1 confirmed marked constitutive uptake of ^{125}I-labeled antibody in lungs and other highly vascularized tissues [44,84].

In agreement with known fact of stimulation of endothelial ICAM-1 synthesis by cytokines, several groups have reported that administration of endotoxin in rats leads to elevation of the vascular binding of ^{125}I-mAb 1A29 and thus stimulates its accumulation in the lung [108]. Elevated pulmonary uptake of ^{125}I-mAb 1A29 has also been described in rats with acute lung injury induced by intratracheal deposition of IgG-immune complexes [110], TNFα [12,108,131], or systemic injection of oleic acid [147]. Cytokine-induced up-regulation of the pulmonary uptake of anti-ICAM-1 ^{125}I-mAb 1A29 requires several hours after LPS injection and reaches peak level at 9-12 h [131]. In mice, TNFα caused most significant increase in the vascular uptake of ^{125}I-mAb 1A29 in the heart and small intestine [44]. Taken together, these results imply that ICAM-1 antibody may serve for the immunotargeting to the inflammation-engaged ('activated') endothelium.

The literature data on ICAM-1 internalization are fragmentary. Results published by several groups imply that endothelial cells in culture poorly internalize antibodies to ICAM-1 [5,85]. This indicates that, unless modification(s) of the carrier antibody facilitate internalization, ICAM-1 is more useful for the surface immunotargeting. While this restricts ICAM-1 potential for delivery of antisense nucleotides, genes or antioxidants, lack of internalization may be very useful for targeting of anticoagulants or fibrinolytics, i.e. drugs which must work on the luminal surface of endothelium.

PECAM-1 (platelet-endothelial cell adhesion molecule-1, CD31) is a 130-kD vascular cell adhesion molecule that is a member of the immunoglobulin superfamily constitutively expressed in the endothelial

cells [2,128]. Endothelial cells possess up to several millions of binding sites for PECAM antibodies per cell, i.e. an order of magnitude more than for ACE, TM or ICAM-1 antibodies. PECAM antibodies provide intense immunostaining of the pulmonary vasculature, as well as vascular cells in other organs. PECAM-1 has very characteristical localization in the intercellular junctions in endothelial monolayer, plays an important role in leukocytes transmigration through the monolayer and contributes to signal transduction in endothelium [128]. Although cytokines cause redistribution of PECAM-1 on endothelial surface in cell culture [140,155], they have no marked effect on PECAM-1 vascular staining in vivo [165]. Notably, TNF does not induce significant changes in biodistribution of radiolabeled PECAM antibody in rats [44]. Although PECAM-1 potential as a target molecule has not been specifically addressed in the literature, this issue certainly worth experimental exploration. Our preliminary data suggest that PECAM-1 mAb may be useful for targeting to the pulmonary endothelium (Muzykantov, Christofidou-Solomidou, Harshaw, Schultz, Fisher and Albelda, in preparation).

By steric masking of their antigens, antibodies to ICAM-1 and PECAM-1 (and Fab-fragments of these antibodies) can block leukocytes adhesion to endothelium and their transmigration into the tissues. This effect of antibodies provides protection of tissues against leukocyte-mediated injury. There are numerous publications reporting protective effect of ICAM-1 and PECAM-1 antibodies in animal models of lung injury caused by cytokines, immune complexes, ischemia reperfusion activation of complement, or lung transplantation [40,41,75,109,165,173]. Blocking of the surface adhesion molecules with antibodies emerged as a novel therapeutic concept and nowadays comes into the clinical practice [75]. Therefore, speculatively, a 'side-effect' of the immunotargeting to the endothelial surface adhesion molecules may provide a secondary benefit for therapy, i.e. blockage of leukocytes adhesion emigration and protection of endothelium. This unique feature, as well as effective targeting to the inflammation-engaged endothelium, make the surface adhesion molecules-directed immunotargeting especially attractive for treatment of disease conditions associated with the vascular inflammation and leukocyte-mediated injury.

3.4. Inducible surface adhesion molecules

In addition to adhesion molecules constitutively expressed on the endothelial membrane, there are specific adhesion determinants (E-selectin, P-selectin and vascular cell adhesion molecule-1) which appear only on the surface of endothelial cells challenged with cytokines, oxidative stress and other pathological factors [2,14,134,154]. Speculatively, utilization of these targets may provide specific recognition of and drug targeting to the inflammation-engaged endothelial cells.

E-selectin is not expressed in the resting endothelium, but cytokines (TNFα, IL-1 in combination with IFγ) and oxidative stress cause up-regulation of its synthesis and expression on the apical surface of endothelium [14]. E-selectin is a single-chain glycoprotein of ≈ 115 kD, serving as counterpart for sialylated Lewis X oligosaccharides on leukocytes and thus contributes to leukocytes adhesion to activated endothelium [45].

Recently, Granger and co-authors, as well as other groups, have studied biodistribution of ^{125}I-mAb to E-selectin and P-selectin in normal and cytokine-challenged rodents, as well as in mice deficient by surface adhesion molecules. ^{125}I-mAb to E-selectin does not bind to vasculature after iv injection in intact rats, but accumulates in many organs several hours after endotoxin challenge, with major uptake in the lungs and mesentery [43]. Interestingly, TNFα-stimulated uptake of ^{125}I-mAb against E-selectin was attenuated in stomach, large intestine and brain of mutant mice deficient by ICAM-1 or P-selectin, although basal level of ^{125}I-mAb against E-selectin uptake in these organs was significantly elevated in ICAM-1-deficient mice vs wild type mice [44]. Intratracheal unilobal administration of endotoxin in rabbits causes local and specific (as compared with control ^{111}In-IgG) uptake of E-selectin ^{125}I-mAb 1.4G2 in the cytokine-challenged, but not in the contralateral lobe [175]. Hascard and co-authors have studied biodistribution of radiolabeled monoclonal antibodies to E-selectin in intact animals and animals challenged with local and systemic inflammatory settings. They reported that:

1. Injection of IL-1 accelerates blood clearance of antibody, thus indirectly indicating its binding to activated endothelium; and
2. According to gamma-immunoscintigraphy, pulmonary uptake of antibody increases after injection of IL-1 [76].

This group also reported enhanced local uptake of radiolabeled antibody to E-selectin in the sites of acute skin or joint inflammation in animal models [72,76,77], as well as in patients with rheumatoid arthritis [25].

P-selectin is stored in Weibel-Palade bodies and absent on the surface of intact endothelium, but cytokines or oxidative stress cause surface exposure of P-selectin on the luminal surface of endothelium [133]. Since the process does not require protein synthesis, P-selectin appears on the surface of endothelium in minutes after the challenge in cell culture. P-selectin has a similar structure to E-selectin, interacts with leukocytes carbohydrates and thus promotes leukocytes adhesion to activated endothelial cells [45,90]. Platelets also contain P-selectin in internal stores and expose it upon activation and, therefore, endothelial immunotargeting by

P-selectin antibody may be confused by platelets activation.

^{125}I-mAb PB1.3 reacting with rat P-selectin accumulates in the rat lungs after intravenous injection of cobra venom factor which causes endothelial activation and injury via complement-mediated pathway [112]. In another study, uptake of ^{125}I-mAb PB1.3 was elevated in the lung and mesentery as soon as 5 min after histamine injection in rats, whereas endotoxin caused more delayed (3-5 h after injection) uptake in the heart and stomach [43]. Interestingly, histamine did not cause vascular targeting of E-selectin antibody in this study, thus suggesting differential regulation of these two selectins in vivo. This notion has been supported by recent study documented that ICAM-1-knockout in mice enhances TNFα-induced up-regulation of the vascular uptake ^{125}I-mAb anti-P-selectin, but rather attenuates that of ^{125}I-mAb anti-E-selectin [44].

Importantly, antibodies against inducible endothelial cell adhesion molecules protect the lung and other tissue against leukocyte-mediated injury associated with various pathological processes. E-selectin antibody blocks leukocytes infiltration in tissues in animal models. P-selectin antibodies inhibit adhesion of leukocytes and attenuate leukocyte-mediated injury in the lung [24,41,107,127] and in heart [87]. Therefore, this side-effect of the immunotargeting directed to P-selectin and E-selectin could provide secondary therapeutic benefit.

Taken together, recent results imply that antibodies to E- and P-selectin could be useful for recognition of and drug targeting to activated endothelial cells. These antibodies worth further exploration as potential imaging, delivering and therapeutic agents. It should be noted, however, that effectiveness of the pulmonary targeting of these antibodies (expressed as percent of injected dose per gram of tissue) is by order of magnitude lower than that of ACE, thrombomodulin and ICAM-1 antibodies. Cellular destination of E-selectin or P-selectin directed immunotargeting has not been addressed in the literature: recent results, however, imply that endothelial cells internalize E-selectin antibody and degrade it in the lysosomal compartment [85]. There is no published data on immunotargeting of drugs using antibodies to E-selectin or P-selectin in animals. Further studies are required in order to estimate diagnostic or therapeutic applicability of the targeting systems based on antibodies to inducible endothelial selectins.

3.5. Other potential endothelial target antigens

Antigens listed in the Table 1 and discussed above are not the only potential endothelial targets. Very attractive methodologies for defining of new prospective targets, based on the selective labeling and consequent separation of proteins localized on the luminal surface of the vasculature of perfused lungs have been described recently by several groups [56,73,100]. A monoclonal antibody recognizing a non-identified endothelial antigen accessible from the bloodstream in the sites of the vascular injury could be useful for the targeting (see below). Preliminary results from laboratory of Dr Jan Schnitzer (personal communication) imply that mAb recognizing a non-identified caveoli-associated antigen could be a good candidate for drug targeting to the pulmonary vasculature. However, both biological functions of these antigens and properties of the carrier antibodies have to be characterized more extensively in order to evaluate the applicability of these pending targeting systems.

4. Immunotargeting of drugs to the pulmonary endothelium

Each targeting system described above offers a unique spectrum of advantages and disadvantages and, therefore, could be more or less appropriate for application at different disease conditions. Antibody-mediated side effects, cellular destination of a drug and effect of inflammatory agents on the targeting are critical parameters for estimation of applicability of the targeting system. Table 2 shows some selected tentative biomedical applications for the immunotargeting of drugs to endothelial cells, with specific respect to the pulmonary vascular endothelium. Noteworthy, certain disease conditions, such as adult respiratory distress syndrome, which involves multicomponent vascular injury in the lung, could be treated by immunotargeting of different drugs (e.g. antioxidants, anticoagulants, fibrinolytics) using different targeting systems (e.g. anti-ACE or anti-CAM).

4.1. Gamma-scintigraphy (radioimmunoimaging)

Many of the published animal studies deal with immunotargeting of isotopes to the vascular endothelium: few human studies on radioimmunoimaging of endothelium have been published [25,121]. The purpose of visualization of the vascular endothelium is to identify defects of the vascular wall, sites of occlusion, restenosis, inflammation, thrombosis or atherosclerosis. With specific focus to the pulmonary vasculature, gamma-imaging may help in diagnostics of pulmonary emboli, lung tumors and inflammation.

Intravenous injection of radiolabeled antibodies against normal endothelial antigens ACE and TM offers visualization of the pulmonary vascular bed in animals [35,79,121] and in humans [121]. As discussed above, injury to the endothelium suppresses pulmonary accumulation of ^{125}I-anti-ACE in animals. Although rather discouraging in terms of the drug delivery, this

Table 2
Potential biomedical applications of the targeting systems described in the paper

Drugs for the targeting	Application	Diseases	Requirements to the targeting system	Potentially applicable system
Radioisotopes	Immunoimaging of embolism, defects of blood flow or vessel wall, vascular occlusions.	Pulmonary embolism (PE), sepsis, ARDS, sarcoidosis, vasculitis.	Recognition of the constitutive EC antigens. Lung selectivity required for PE imaging.	ACE
Radioisotopes	Immunoimaging of inflammation-engaged EC in the lung or in other organs.	Vascular inflammation, sepsis, ARDS, autoimmune diseases, arthritis.	Recognition of inducible adhesion molecules.	E-selectin, P-selectin
Antioxidants	Protection of EC against injury induced by either intracellular (e.g. ischemia) or extracellular (PMN-released) oxidants.	O_2 toxicity, ARDS, sepsis, ischemia/reperfusion, lung transplantation, bleomycin toxicity, vascular inflammation.	Recognition of either normal or inducible antigens. Lung selectivity, inhibition of PMN-mediated injury and internalization of antioxidants are beneficial.	ACE, ICAM-1, other CAM
Anticoagulants	Augmentation of the EC anticoagulant potential.	ARDS, sepsis, DVT, PE, anticoagulant therapy.	Lack of rapid intracellular uptake, i.e. EC surface targeting. Lung selectivity is beneficial for treatment of PE and ARDS.	ICAM-1.
Fibrinolytics	Facilitation of the fibrinolysis. Correction of PAI overexpression in the lung tissue.	PE, ARDS, sepsis, DVT, fibrinolytic therapy.	As above.	As above.
Protease inhibitors	Regulation of the vascular remodelling, angiogenesis and restenosis.	Atherosclerosis, vascular surgery, angioplasties, tumors.	EC surface targeting.	As above.
Genes	Expression of NOS, antioxidant enzymes, plasminogen activators or inhibitors, etc.	Pulmonary and systemic hypertension, oxidative injury, atherosclerosis, thrombosis, restenosis, vascular inflammation.	Internalization and nuclear targeting.	ACE, TM, E-selectin.
Antisense oligonucleotides	Tissue-selective inhibition of EC synthesis of specific protein (CAM, NOS, ACE, PAI, tPA, uPA, uPAr, etc).	As above.	Internalization.	As above.
Toxic agents	Site-specific EC injury by specific mechanism, e.g. immunotargeting of ROS-generating enzyme to model EC oxidative stress.	New animal models of the vascular injury localized in the selected vascular areas.	Tissue selectivity determined by a model. Lack of confusing effects of a carrier antibody.	?

Abbreviations: EC, endothelial cells; ACE, angiotensin-converting enzyme; TM, thrombomodulin; CAM, cell adhesion molecules; ARDS, adult respiratory distress syndrome; PE, pulmonary embolism; DVT, deep vein thrombosis; PMN, plymorphonuclear leukocytes; PAI, plasminogen activator inhibitor; tPA, tissue-type plasminogen activator; uPA, urokinase; uAPr, urokinase receptor; NOS, nitric oxide synthase; ROS, reactive oxygen species.

observation implies that radiolabeled anti-ACE could be useful for detection of endothelial injury in the lungs [118]. A pilot clinical study revealed that pulmonary uptake of ^{99m}Tc-labeled mAb 9B9 is significantly decreased in sarcoidosis patients [121]. Although this preliminary study did not provide an insight into the significance or mechanism(s) of the observed phenomenon, it supports the notion that gamma-imaging of ^{99m}Tc-labeled mAb 9B9 may have a diagnostic value, for detection of endothelial injury or defects of blood flow to the site of interest.

Recently, radiolabeled antibodies to cell adhesion molecules (anti-CAM) also became an interesting tool for exploration in gamma-imaging in animal and human studies. ^{111}In-labeled antibody to ICAM-1 demonstrated high pulmonary uptake visible in a gamma-camera after systemic injection in control rats [148]. The pulmonary uptake of ^{111}In-anti-ICAM-1, however, is detectably increased in acute lung injury associated with transplant rejection [6] and injection of oleic acid [147]. Acute myocardial infarction in rats leads to elevated myocardial uptake of anti-ICAM-1 ^{125}I-mAb, as well as accumulation of anti-ICAM-1 ^{111}In-mAb in the cardiac area, detectable by imaging in a gamma-camera [65]. Enhanced pulmonary uptake of ^{111}In- or ^{99m}Tc-mAb 1.2B6 against E-selectin has been visualized in pigs injected with IL-1 [76]. This antibody also allows visualization of local inflammation in the skin and knee joint in animal models [72,77] and in patients with rheumatoid arthritis [25]. Therefore, immunotargeting of isotopes to the surface adhesion molecules may eventually provide a new diagnostic tool for visualization of inflammation-engaged endothelium in the lung and other organs of interest.

4.2. Delivery of fibrinolytics and anticoagulants

Pulmonary vasculature is susceptible to occlusion by thromboemboli. Pulmonary embolism (PE) is a serious complication of various extrapulmonary syndromes, diseases such as deep vein thrombosis (DVT) and surgery and causes 50000 death per year in USA [66,151]. In addition to PE induced by a single clot, there are several diseases and syndromes associated with disseminated fibrin deposition in the pulmonary vasculature, such as sepsis and ARDS [62,158]. Fibrin deposition and plasmin suppression in the lung contribute to the development of pulmonary fibrosis and hypertension [42,105]. Therefore, site-specific prevention, detection and treatment of the pulmonary deposition of fibrin and emboli represent an important biomedical problem. As discussed above, immunotargeting of isotopes to the vascular endothelium may offer a new approach for detection of the vascular occlusions and pulmonary embolism.

Systemic administration of anticoagulants, such as heparin, is currently a most useful method for prevention of PE [151]. Plasminogen activators, such as urokinase or tissue-type plasminogen activator (tPA) are clinically useful fibrinolytics; however, they do not have an affinity to endothelium and undergo fast inactivation and elimination from the bloodstream [88,168]. Plasminogen activators, therefore, are used for treatment of PE on a limited scale, because of high probability of extrapulmonary bleeding, modest effectiveness of dissolution of pulmonary emboli and high probability of re-occlusions [66,137,169,151]. Conjugation of plasminogen activators with fibrin-specific antibodies, as well as creation of gene engineered mutant proteins may help to improve the therapy [88]. On the other hand, delivery of anticoagulants and fibrinolytics to endothelial cells (especially in the lung) may be useful for either reduction of fibrin formation or facilitation of its degradation. Local delivery of the conjugates via vascular catheters may provide an additional augmentation of the specificity and effectiveness of the therapy.

There are two major requirements for targeting system for delivery of either anticoagulants or fibrinolytics to endothelium. First, internalization of the conjugate should be minimal, in order to maintain an active drug on the luminal surface. Second, carriers which may compromise endothelial anticoagulant activity (e.g. anti-thrombomodulin), must be excluded from the consideration.

Anticoagulant hirudin has been conjugated with E-selectin antibody and experiments in endothelial cell culture have documented that the conjugate binds specifically to cytokine-activated endothelium and inhibits thrombin [82]. However, applicability of E-selectin as targeting system is questionable because: (i) it will not deliver anticoagulant to the normal endothelium (thus precluding preventive treatment); and (ii) endothelial cells internalize E-selectins. Urokinase has been conjugated with a monoclonal antibody recognizing non-identified 75-kD intracellular antigen which is absent on the surface of an intact endothelium, but becomes accessible to the bloodstream in the sites of endothelial injury [138]. This conjugate has been shown to reduce thrombotic events in the pre-treated vena cavae graft in rats [15,103,164]. Conjugation of plasminogen activators (tPA, urokinase, streptokinase) with the carrier anti-ACE antibody provides the conjugates which bind to the immobilized antigen and cause local fibrinolysis in the antigen-coated wells in vitro, accumulate in the rat lungs after intravenous injection and are retained in the lung tissue for at least several hours [123]. However, fibrinolytic activity of these conjugates in the lungs, as well as the fate of the conjugates and their potential side effects remain to be studied. Speculatively, anti-ICAM-1 carriers seem to be more appropriate for immunotargeting of anticoagulants or fibrinolytics, be-

cause endothelium poorly internalizes this constitutively expressed surface adhesion molecule.

4.3. Antioxidative protection

Endothelium is vulnerable to oxidative injury caused by oxidants, either extracellular (e.g. released from activated leukocytes, [49,54]) or intracellular (e.g. generated by endothelial cells challenged with cytokines or upon ischemia reperfusion [50,98]). Endothelial cells in the lungs are particularly susceptible to oxidative injury, because: (i) oxygen and oxygen metabolites tension in the lung tissue is very high; and (ii) and pulmonary vasculature is a target for leukocytes activated in the circulation [17,52,93]. Two antioxidant enzymes, superoxide dismutase (SOD, that converts superoxide anion to hydrogen peroxide) and catalase (that decomposes H_2O_2 to water) have been extensively explored as potential drugs for antioxidative protection of the endothelial cells [1,13,53,70,157]. Despite a great deal of effort, however, antioxidative protection of vascular endothelium utilizing any known derivative of SOD or catalase remains below an acceptable level of effectiveness and specificity [59]. This may be explained by the lack of: (i) antioxidants targeting to the endothelial cells; and (ii) proper intracellular addressing of antioxidants. Therefore, immunotargeting of SOD and or catalase may help to solve the problem.

A decade ago, we have described that catalase conjugated with anti-endothelial antibody binds to endothelium in culture and protects it against oxidative injury induced by H_2O_2 [113,146]. Polyclonal antibodies utilized in the cited studies, however, did not distinguish between endothelial and blood cells and thus were not applicable for in vivo studies. In order to improve the specificity, we have conjugated ^{125}I-catalase and ^{125}I-CuZn-SOD with anti-ACE mAb 9B9 and have documented that:

1. mAb 9B9-conjugated antioxidants accumulate in the pulmonary vasculature after iv injection in rats (8% of dose/lung vs. 0.5% for IgG-conjugated enzymes).
2. EC internalize catalase conjugated with mAb 9B9; and
3. Both SOD and catalase conjugated with mAb 9B9 are retained in the lung for a prolonged time after injection [124].

In a recent study we have shown that catalase conjugated with anti-ICAM mAb 1A29 also accumulates in the pulmonary vasculature and that both anti-ACE/catalase and anti-ICAM/catalase can blunt oxidative injury caused by exogenous H_2O_2 in the perfused rat lung [9]. These results imply that immunotargeting of antioxidants may provide a novel strategy for the therapy, although experiments in more pathophysiologically relevant animal models (hyperoxia, sepsis, bleomycin-induced lung injury) must be performed to prove validity and feasibility of the strategy.

4.4. Targeting of liposomes

Liposomes may be utilized as a universal vehicle for drug targeting [3,61,91,135]. Importantly, encapsulation of a drug in liposomes provides its protection from inactivation in the bloodstream, as well as facilitation of the intracellular delivery [53]. Stealth-liposomes, developed recently, have prolonged half-life in the circulation and accumulate in the target tissues after intravascular administration [3]. Although targeting of liposomes to the extravascular targets is strongly restricted by slow rate of their transfer through the tissue barriers, endothelium represents particularly attractive target for immunoliposomes because of its high accessibility to the bloodstream.

In 1988, Torchilin and associates have reported specific binding and internalization of liposomes coated with mAb E25 (an antibody that recognizes a non-identified endothelial antigen) in endothelial cell culture [160]. Since then, several papers published by this and other groups have documented that liposomes conjugated with thrombomodulin monoclonal antibody accumulate extremely effectively and selectively in the lungs after iv injection in mice [67,96,97]. More recently, anti-TM carrier has also been used for immunotargeting of microspheres: about 70%ID/g of immunomicrospheres was localized in the murine lungs 1 h after iv injection; target cells are capable of internalization of immunomicrospheres [163]. Therefore, liposomes and microspheres may be useful for delivery of drugs or genes to the pulmonary endothelum, although potential functional therapeutic effects of this approach remain to be characterized.

4.5. Gene delivery

Gene therapy could theoretically offer a way to express genes encoding therapeutic proteins in the target cells [58,92]. This strategy has been tested in cell culture experiments where transfection of cells with genes encoding antioxidant proteins led to augmentation of antioxidant defenses in the target cells [47,152,170]. Local administration of polycationic lipid:DNA complex and adenoviruses via intrapulmonary catheter has been attempted in an effort to express reporter genes in pulmonary endothelium [106,143]. Unfortunately, at this time, effective, high level gene transfer to vascular endothelium has not been achieved [30,149]. In addition, even if sufficient gene transfer did occur, protein synthesis requires substantial time. Therefore, gene transfer would not likely be useful for the treatment of acute pulmonary or vascular disorders when rapid action is required.

Conjugation of DNA or DNA-loaded liposomes with carrier antibody may provide both targeting and intracellular uptake of DNA. Trubetskoy and co-authors [161,162], have conjugated the carrier thrombomodulin antibody with either cationic liposomes loaded with reporter DNA or with DNA directly, via polylysine cross-linker. Injection of these conjugates in mice led to significant elevation of activity of the reporter proteins (CAT) in the lung tissue [161,162]. Although these results are encouraging, cell-selective immunotargeting of genes to the pulmonary endothelium is still at its very early infancy. Several fundamental issues remain to be addressed. First, intracellular uptake and nuclear trafficking of the conjugates should be attained. This circumstance raised specific requirements to the carrier or and its modification (e.g. insertion of nuclear leading signal). Based on the information available, anti-ACE mAb 9B9 looks promising for intracellular delivery. Second, effectiveness of transfection must be enhanced. Insertion of endothelium-specific promoters (e.g. thrombomodulin promoter) in the gene construct may help to enhance the tissue selectivity of transfection. It is tempting to speculate that eventually, the combination of enzyme immunotargeting (that could provide rapid but transient effect) with gene transfer (that could provide delayed but prolonged effect) could be useful for treatment of endothelial injury.

4.6. Targeting of cytotoxic agents and oxidants

Immunoselective delivery of injuring agents may represent a new methodology for modeling of the endothelial vascular injury. Antibiotic-loaded liposomes conjugated with E-selectin mAb bound selectively to cytokine-activated, but not to the control endothelial cells in culture and, furthermore, provided intracellular delivery of antibiotic and killed the target cells [153]. Although practical applicability of this conjugate is questionable, it demonstrates high selectivity of this targeting system, potentially applicable for delivery of other drugs and agents to the inflammation-engaged endothelium.

H_2O_2-generating enzyme, glucose oxidase (GOX) has been conjugated to various polyclonal and monoclonal antibodies reacting with endothelial antigens [117]. Properties of these conjugates have been characterized in vitro and in vivo with the following results: (i) anti-EC/GOX binds to cultivated endothelium, generates H_2O_2, and kills cells [114]; (ii) anti-ACE/GOX circulates in the blood as an entire complex and accumulates in the rat lungs after iv injection [115], and (iii) GOX conjugated with internalizable antibody causes more severe oxidative stress in endothelial culture, as compared with non-internalizable counterpart [116]. These results imply that antibody-conjugated glucose oxidase is useful as a tool to study intracellular oxidative stress in the endothelium.

Importantly, cytotoxic agents, pro-coagulation factors, toxins or glucose oxidase conjugated with antibodies recognizing endothelial cells localized in the tumors, may represent a novel avenue for treatment of solid tumors. Very recently, stealth-liposomes conjugated with antibody against thrombomodulin have been utilized for targeting of lipophilic toxic prodrug to the pulmonary microvaculature in mice bearing pulmonary vascular tumors and significant increase in survival of tumor-infected mice has been reported [104]. This result implies that generation of high local concentration of a prodrug in the target organ may enhance effect of chemotherapy, albeit safety of such semi-specific immunotargeting must be carefully tested. Certainly, tumor-specific endothelial antigens are preferential for immunotargeting of chemotherapy [20].

5. Safety concerns

Although anti-endothelial Ab may provide the targeting, it also may cause serious side effects. Endothelium-associated Ab may activate complement and/or leukocytes via Fc-fragment-mediated pathways. For example, heterologous polyclonal anti-ACE Ab have been reported to cause an acute lung injury mediated by complement and leukocytes [10,22,23]. Therefore, antibodies to be useful as carriers must not fix complement or activate leukocytes. Although some isotypes of immunoglobulins naturally lack these functions, utilization of Fab fragments seems to be obligatory for the targeting.

Previous results demonstrated that animals tolerate well injections of a large doses of anti-ACE [37,38] and antibodies to the surface adhesion molecules [24,41,87,107,127,165]. Nevertheless, at the present time, immunotargeting can not be considered as a therapy for prolonged or multiple administration because of obvious immune restrictions: only further progress in the development of the conjugates will grant their wide clinical applicability (see below).

Antibodies may alter membrane metabolism of antigens in endothelial cells. For example, thrombomodulin antibodies stimulate its internalization [18], whereas ACE antibodies stimulate its shedding from the plasma membrane [38]. Cross-linking of antigen molecules in the plasma membrane by antibodies also may cause significant alterations in endothelial physiology, for example, stimulate signal transduction and elevate intracellular Ca^{2+}. In addition, either antibody or conjugates may mask antigens and thus inhibit their activity in the bloodstream. Therefore, each targeting system must be carefully tested for more specific, i.e. an antigen-related safety aspects. For example, targeting to ACE must be carefully studied in terms of possible effects mediated by ACE inhibition (suppression of

angiotensin II production and protection of bradykinin). Although inhibition of the CAM generally is considered as a benefit for the therapy, in some cases this effect may compromise normal immune response to infection.

Every targeting system must be carefully tested for potential side-effects of high local concentration of the targeted drug. For example, safety of application of antibody-conjugated plasminogen activators must be characterized in terms of their effect on extracellular matrix and integrity of the vascular wall. Generally speaking, overloading of the target cells with any drug or gene, even relatively safe ones, may cause endothelial exhaustion, dysfunction and injury.

6. Future perspectives

In musical terms, progress attained in immunotargeting to endothelium in the last decade of the century could be characterized as an 'intermezzo' between an entirely speculative concept and a clinically applicable therapeutic strategy. Several potentially useful antigens and set of carrier antibodies have been defined. Certainly, new targeting systems will evolve, including those recognizing specific vascular areas more precisely than existing antibodies. Further steps will identify the most effective targeting systems for various applications and characterize local and systemic effects of the conjugates in animal models. Chemical modification of the conjugates (e.g. incorporation of nuclear-leading signals), will provide more precise intracellular addressing of a drug. Utilization of small antigen-binding fragments of antibodies and their humanization will be necessary for clinical practice, in order to exclude Fc-portion mediated effects and immune response against murine immunoglobulins, whereas chimerical proteins which consist of the hypervariable domain or minimal recognizing unit (MRU) and an effector enzyme or its active site could be created by gene engineering technology [88,130,139]. This will allow experiments in primates, clinical trials and evaluation of the therapeutic potential of the endothelial immunotargeting of drugs. Certainly, translation from the laboratory experiments to the bedside will require a large investment and efforts. It would be quite unrealistical to expect this therapeutic strategy to be utilized in the clinical practice in this millennium, but the next millennium is coming very soon.

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BIOMEDICAL ASPECTS OF DRUG TARGETING

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TABLE OF CONTENTS

SECTION 1: GENERAL PRINCIPLES OF DRUG TARGETING

SECTION 2: CARDIOVASCULAR TARGETING

7

TARGETING PULMONARY ENDOTHELIUM

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INTRODUCTION

The pulmonary endothelium maintains vital functions including control of thrombosis, inflammation, vascular permeability and blood pressure. Endothelial cells in the lung blood vessels are vulnerable to oxidative, thrombotic and inflammatory insults and represent an important target for therapies. However, despite the huge surface and high accessibility of this target to blood, only a minor fraction of circulating therapeutics is adequately delivered to the pulmonary endothelium. Effective, safe and specific delivery of drugs, enzymes and genetic materials to this target may help to improve current modalities in therapies and prophylaxis of pulmonary hypertension, oxidative stress, embolism, edema, acute lung injury, and other disease conditions.

Affinity carriers, such as monoclonal antibodies and endothelial surface determinants, may permit targeting and proper sub-cellular addressing of drugs in the pulmonary vasculature. Vascular immunotargeting may also provide secondary therapeutic benefits due to blocking of the target antigens, such as surface adhesion molecules involved in inflammation or angiotensin-converting enzyme involved in hypertension. This chapter describes features of the pulmonary endothelium as a therapeutic target, outlines principles, methodologies, the current status of vascular immunotargeting to the pulmonary endothelium, and gives specific examples of delivery systems that show promising results in pre-clinical animal studies.

Pulmonary Endothelium as a Therapeutic Target

Pulmonary endothelial cells, as well as the endothelium in other organs, form a cellular monolayer on the luminal surface of blood vessels and control vascular permeability, blood-tissue exchanges, blood pressure and fluidity, interactions between blood cells and tissues, as well as other important normal and pathological functions of the vascular system.

The endothelium produces and controls synthesis of agents that cause either vasorelaxation (e.g., nitric oxide, NO, and prostacyclin) or vasoconstriction (peptides, endothelin, angiotensin II, Ang II). Peptidases and proteases exposed on the surface of endothelial cells activate vasoactive propeptides. For example, angiotensin-converting enzyme, ACE, converts Ang I into Ang II and inactivates bradykinin and substance P (Erdos et al., 1990). Thus, blood perfusion through the lungs serves both gas exchange purposes and enables the conversion of numerous peptides that regulate blood pressure and vascular permeability.

The endothelium helps to prevent thrombosis. In concert with plasma protein C, endothelial transmembrane glycoprotein thrombomodulin converts thrombin into an anti-coagulant enzyme (Esmon, 1989). Among other antithrombotic factors, endothelium secretes NO that suppresses platelet aggregation as well as urokinase and tissue type plasminogen activators that dissolve blood clots via generation of a fibrin-degrading protease plasmin.

The endothelium controls inflammation, a process that is often intertwined with thrombosis. Under normal circumstances, endothelial cells provide very limited, if any, support for activities of pro-inflammatory cells (e.g., white blood cells). However, pathological mediators, including cytokines, reactive oxygen species, growth factors and abnormal shear stress, induce endothelial secretion of chemoattractants and exposure of adhesion molecules leading to leukocyte attraction, adhesion and transmigration (see schema of vascular inflammation in Chapter 2).

Endothelial cells play an important role in normal and pathological vascular redox mechanisms. They produce reactive oxygen species (ROS) including superoxide anion (O_2^-) and H_2O_2 via enzymatic pathways that can be further activated by pathological mediators. ROS apparently play an important role in cellular signaling. However, their overproduction leads to vascular oxidative stress, inactivation of NO by O_2^-, lipid peroxidation and tissue injury (Freeman and Crapo, 1987).

In the lungs, most of endothelial cells belong to the alveolar capillaries that are constitutively exposed to relatively high oxygen levels and

vulnerable to pro-inflammatory and damaging effects of alveolar macrophages activated by inhaled agents (e.g., dust and pathogens). Pulmonary vasculature is a target of pathological remodeling and fibrosis associated with many forms of heart diseases, drug toxicity, radiation injury and primary pulmonary hypertension. Alveolar capillaries represent a filter, collecting activated leukocytes, metastasizing tumor cells and clots from the venous blood.

The pulmonary endothelium is affected by hyperoxia, hypertension, smoke, dust inhalation, pneumonia, cardiac failure, sepsis, thrombosis, diabetes, cancer and many other pulmonary and systemic disease conditions. Endothelial injury leading to a vicious cycle of pulmonary thrombosis, inflammation and edema underlies the pathogenesis of some forms of Acute Lung Injury (ALI) or Adult Respiratory Distress Syndrome (ARDS), a life-threatening condition associated with trauma, sepsis and hemorrhage that cause a high mortality.

Theoretically, adequate (i.e., effective, specific, safe and directed to a proper sub-cellular compartment) delivery of therapeutics (e.g., drugs, proteins or genes encoding these proteins) could improve treatment of these disease conditions. For example, targeting of NO-donors, NO-synthase or gene encoding NOS could be useful for treatment of pulmonary hypertension. In addition, targeting of anti-oxidant and anti-inflammatory agents might help to manage pulmonary oxidative stress and inflammation, whereas targeting of anti-thrombotic agents might prevent thrombosis or facilitate dissolution of thrombi.

Immunotargeting to the Pulmonary Endothelium: An Overview

The pulmonary vasculature is the first major capillary network encountered by drugs after intravenous injection. It contains roughly one third of endothelial cells in the body, filters the whole cardiac output of venous blood, and represents a privileged target for vascular drug delivery (Danilov et al., 2001). Thus, cationic liposomes and many viruses accumulate in the lungs due to blood filtration. It should be noted, however, that the specificity and safety of this strategy remain to be more rigorously characterized.

Unless drugs are coupled to high-affinity carriers, even after local infusion via vascular catheters, the perfusion removes drugs and their derivatives rapidly. Devices allowing a transient cessation of blood flow in the site of catheter placement have been designed in order to attain a high local concentration and a more effective prolonged interaction of the infused

material with endothelium. However, blood flow interruption may lead to ischemia and vascular injury. The lack of a viable means for the effective, rapid, and safe targeting of therapeutic molecules to the endothelium represents an important biomedical problem.

Vascular immunotargeting, a strategy utilizing coupling of drugs to antibodies or antibody fragments to endothelial determinants, promises such a means of delivery of drugs and genetic materials to the pulmonary endothelium (Muzykantov, 1998). The requirements that should be met in order to achieve immunotargeting to pulmonary endothelium include the following:

- Target antigens should be abundant on the endothelium to permit robust targeting. When a transiently expressed antigen is used as a target, the identified time window should be adequate for drug delivery.
- Target antigens should not be present in blood or in non-endothelial cells that are accessible to blood. For example, endothelial cells have transferin receptors; however, they are also abundantly expressed in hepatic and other cells accessible to blood. Therefore, transferin-targeted drugs accumulate mostly in the liver (and, to some extent, in the brain, since cerebral endothelium is enriched in transferin receptors), with a relatively modest uptake in lungs. Plasma or blood cells' antigens' drug delivery detracts from delivery to the endothelium.
- The level of antigen expression by endothelial cells should not be suppressed upon conditions at which targeting is desirable.
- Depending on the therapeutic goal, an antibody-drug complex should be either retained on the cell surface or undergo trafficking to a proper subcellular compartment.
- The binding of antibody-drug complexes to an antigen should not cause harmful or uncontrolled side effects.
- Ideally, binding of an antibody-drug complex to a target antigen should cause therapeutically beneficial side effects.
- Theoretically, recognition of specific types of endothelial cells and even certain parts of an endothelial cell body is possible.

The results accumulated within the last decade indicate that monoclonal antibodies to angiotensin-converting enzyme, surface adhesion molecules, and caveoli-associated antigens represent potentially useful carriers for immunotargeting pulmonary endothelium. In the following sections, we briefly discuss methodology of the studies and several selected examples of the immunotargeting.

Methodological aspects of the vascular immunotargeting

Several experimental models are used to study targeting of the pulmonary endothelium. Endothelial cell cultures provide a useful model to determine the affinity of binding, mechanisms of intracellular uptake, and targeting effects under defined conditions with practically unlimited variables and number of measurements. Tracings of radiolabeled compounds, as well as fluorescent and electron-microscopy imaging, provide abundant and easily interpretable data (Figure 1, below). However, the identity of the cultivated cells to their counterparts *in vivo* is questionable (especially at high culture passages) and cells in culture lack adequate environmental factors such as extracellular matrix, physical stress, as well as neuronal and hormonal regulation.

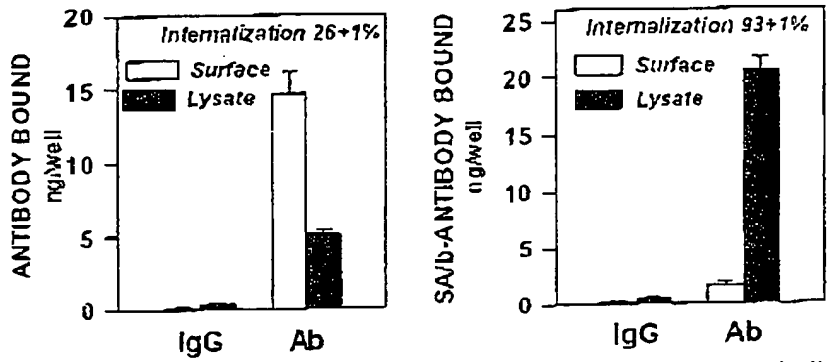

Figure 1. Binding and internalization of radiolabeled PECAM-1 monoclonal antibodies (left panel) and anti-PECAM streptavidin conjugates (SA/b-antibody, right panel) in human umbilical vein endothelial cell cultures (HUVEC). Analysis of the surface-associated and internalized radioactivity was performed using acidic glycin elution. Anti-PECAM and anti-PECAM/SA (both indicated as Ab), but not IgG counterparts specifically bind to the endothelial cells, which constitutively express PECAM. Anti-PECAM remains mostly associated with the cell surface whereas SA/b-anti-PECAM conjugate is internalized at 37°C (Adapted from Muzykantov et al, PNAS USA 1999, with permission, see comments in the section describing PECAM targeting).

Perfusion of isolated lungs represents a unique model that eliminates many artifacts of cell cultures and permits the use of diverse perfusion and ventilation conditions (variable flow rate, temperature, gases, and blood components) to study interactions of circulating agents with pulmonary endothelium in the absence of confusing systemic effects. This model allows confirmation of the specificity and kinetic parameters of the targeting, as well as identification of subcellular addressing of cargoes and effects of the targeting (Muzykantov et al., 1999; Danilov et al., 2001; see also Chapter 6 by Carver and Schnitzer).

Animal studies ultimately evaluate targeting under normal or pathological states. Injections of a mixture of immune and non-immune conjugates labeled with different isotopes (e.g., ^{125}Iodine and ^{131}Iodine) permit the most accurate measurement of several parameters of targeting (Figure 2). Percent of injected dose uptake in an organ (%ID) shows biodistribution in the body and robustness of delivery to a target organ. Secondly, %ID normalized per gram of tissue (%ID/g) permits comparison of uptake in different organs and tissue selectivity of the uptake. This parameter also permits comparison of the data obtained in different animal species. Ratio between %ID/g in an organ and in blood gives the Localization Ratio, LR. This parameter compensates for a difference in blood level of circulating radiolabeled antibody (e.g., due to different rate of uptake by clearing or target organs). The Immunospecificity Index (ISI) ratio of the pulmonary uptake of immune and non-immune counterparts provides the most objective measure of targeting specificity.

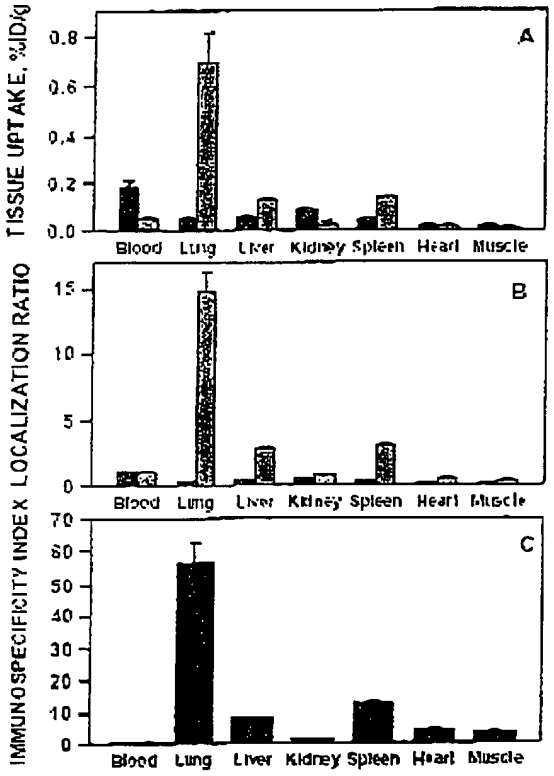

Figure 2. Distribution of ^{131}I-IgG (closed bars) and anti-PECAM/^{125}I-streptavidin (hatched bars) in the organs 1 hr post-systemic intravenous injection in pigs. A. Absolute values of the isotopes uptake normalized per weight. B. Ratio of radioactivity per gram of tissue to that in blood (Localization Ratio). C. Immunospecificity Index of anti-PECAM uptake in the organs calculated as ratio of LR for ^{125}Iodine and ^{131}Iodine. Note that the anti-PECAM conjugate, but not the non-immune control counterpart, preferentially accumulates in the lungs (Adapted from Scherpereel et al., JPET 2002, with permission).

SELECTED EXAMPLES OF PULMONARY TARGETING

No universal or ideal carrier that suits all therapeutic needs exists. Specific therapeutic goals require different secondary effects mediated by binding to the target, drug targeting to different populations of endothelial cells (e.g., resting *vs.* inflammation-engaged), and diverse cellular compartments. Table 1 shows endothelial determinants useful for experimental and, perhaps, therapeutic targeting pulmonary endothelium.

Table 1. Endothelial determinants: candidate targets. EC – endothelial cells, ACE – angiotensin-converting enzyme, TM - thrombomodulin, PECAM - platelet-endothelial adhesion molecule, ICAM - intercellular adhesion molecule; gp - glycoproteins.

Target	Function and Localization	Targeting Advantages	Potential Problems
ACE	Peptidase, converts Ang I into Ang II and cleaves bradykinin. ACE enriched in the lung capillaries.	Selective targeting to lung EC. Intracellular delivery. Vasodilating and anti-inflammatory effects of ACE inhibition.	Inflammation suppresses targeting. ACE inhibition may be dangerous.
TM	Binds thrombin and converts it into anti-coagulant enzyme. Enriched in the lungs.	Intracellular delivery to EC useful for modeling of lung injury in animals.	Inflammation suppresses targeting. Thrombosis due to TM inhibition.
PECAM	Facilitates transmigration of leukocytes. Stably expressed in EC borders.	Intracellular delivery of anti-PECAM conjugate may also suppress inflammation	PECAM-signaling and side effects are not understood
ICAM	Mediates leukocyte adhesion to EC. Stably expressed by EC, and up-regulated by pathological agents.	Similar to PECAM, but inflammation enhances targeting.	Similar to PECAM
E-selectin	Supports leukocytes adhesion. Expressed only on altered EC.	Intracellular targeting to EC in inflammation	Targeting is not robust. Transient expression.
P-selectin	Similar to E-selectin	Similar to E-selectin	Similar to above. Targeting platelets
gp90	Function unknown. Localized in EC cavoli.	Transendothelial targeting.	Human analogue and side effects are not known.
gp95	Function unknown. EC avesicular zone in alveolar capillaries.	Targeting to the EC surface	Similar to above
gp60	EC caveoli	Untested	Unknown

Angiotensin-converting enzyme (ACE)

Angiotensin-converting enzyme, a transmembrane glycoprotein expressed on the luminal surface of endothelial cells, is one of the key components of the renin-angiotensin system. Importantly, pulmonary vasculature is markedly enriched in ACE; nearly 100% endothelial cells in the alveolar capillaries are strongly positive for ACE, whereas only 10-15% of endothelial cells in the extra-pulmonary capillaries are ACE-positive (Danilov et al., 2001). In late eighties, Sergei Danilov and co-authors proposed utilizing anti-ACE as an affinity carrier for drug targeting to the endothelium and showed that radiolabeled anti-ACE accumulates selectively in the pulmonary vasculature after intravascular and intraperitoneal injections in rats, hamsters, cats and primates (Danilov et al., 1991; Muzykantov and Danilov, 1995). The following studies showed that endothelial cells internalize anti-ACE that may be used for intracellular drug delivery (Muzykantov et al., 1996a). Diverse reporter compounds and drugs (e.g., antioxidant enzyme catalase) conjugated with anti-ACE accumulate selectively in the lungs after intravenous injection in rats (Muzykantov and Danilov, 1995; Muzykantov et al., 1996b).

ACE converts Ang I into Ang II (Erdos, 1990), a potent vasoconstricting peptide that also has pro-oxidant and pro-inflammatory activities. On the other hand, ACE inactivates bradykinin, a peptide stimulating endothelial production of NO (see Figure 3 below), an important vascular mediator that causes relaxation of vascular smooth muscle cells and suppression of platelet aggregation. Superoxide anion produced by endothelial cells in response to pro-inflammatory agents (including Ang II), inactivates NO and generates peroxinitrite, a potent oxidant. Therefore, inhibition of ACE by anti-ACE causes vasorelaxation and attenuates inflammation and oxidative stress. This effect represents a potential secondary benefit of ACE immunotargeting in certain pathological settings. In patients with acute hemorrhaging and hypotension, this effect may be rather dangerous due to a high probability of causing vascular collapse.

Some ACE antibodies are inhibitory due to the blocking of the active site; yet even non-inhibitory antibodies may suppress ACE activity in the endothelium due to facilitation of its shedding from the plasma membrane. The latter effect is epitope-specific and varies for different monoclonal antibodies (Danilov, 1994; Balyasnikova et al., 2001). Theoretically, ACE antibodies against diverse epitopes may be used for either ACE-inhibiting or non-inhibiting immunotargeting of drugs, thus providing an additional flexibility to the strategy and further advancing its therapeutic applicability.

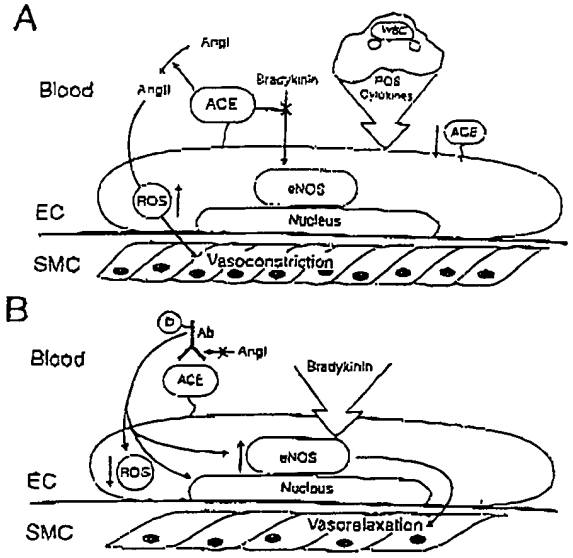

Figure 3. Immunotargeting to ACE (see explanations in the text). EC – endothelial cells, SMC – smooth muscle cells, eNOS – endothelial NO synthase, WBC – white blood cells; ROS – reactive oxygen species, Ab – monoclonal antibody (anti-ACE) conjugated with drugs (D). Panel A: ACE is normally expressed on the endothelial surface (left part), but inflammatory factors (e.g., ROS and cytokines released from activated leukocytes) down-regulate ACE surface density on endothelium (right part). Panel B: Anti-ACE delivers drugs to endothelium and undergoes internalization. This paradigm can be utilized for delivery of antioxidant enzymes (to intercept intracellular ROS) or genetic materials. In addition, anti-ACE inhibits ACE. This attenuates Ang I conversion, protects bradykinin (both of these effects lead to vasorelaxation), and suppresses pro-oxidative effects of Ang II.

Potential therapeutic applications of ACE targeting include intracellular delivery of active enzymes and genetic materials. For example, anti-ACE-conjugated catalase, an antioxidant enzyme that degrades H_2O_2, has been shown to accumulate in the perfused rat lungs and protect the pulmonary endothelium against oxidative stress (Atochina et al., 1998). Recently, ACE immunotargeting has been used for re-targeting of viruses to the pulmonary endothelium (see Chapter 9 by Reynolds and Danilov).

ACE immunotargeting is highly tolerable; anti-ACE does not induce acute harmful reactions in laboratory animals and human volunteers (Muzykantov and Danilov, 1995). One potential problem associated with targeting ACE is that endothelial expression of ACE is suppressed upon many pathological states, including hyperoxia, inflammation, oxidative stress and sepsis (likely due to effects of the cytokines, ROS and other pro-inflammatory factors) (Muzykantov and Danilov, 1995). This effect may compromise the robustness of therapeutic targeting to ACE. However, ACE is one of the

premier candidate targets for drug delivery to the pulmonary endothelium, for example, for a prophylactic use.

Platelet-Endothelial Cell Adhesion Molecule (PECAM-1)

PECAM is a pan-endothelial, immunoglobulin superfamily transmembrane glycoprotein, which is predominantly localized in the sites of cellular contacts in the endothelial monolayer. Platelets and white blood cells also express PECAM, but in lesser quantities. The larger portion of the PECAM molecule (extracellular domain) in endothelial cells is exposed to the lumen and serves as a counterpart for leukocytes, thus supporting their transendothelial migration in the sites of inflammation (Newman, 1997). In addition to this function, as documented in many experimental models (Vaporcyian et al, 1993), PECAM apparently may be involved in endothelial signaling and activation, but this aspect of its function remains to be better understood (Newman, 1997).

PECAM is abundant in endothelial cells that express millions of binding sites for anti-PECAM (Muzykantov et al., 1999). In addition, PECAM is a stable endothelial antigen; cytokines and ROS do not down-regulate its expression and surface density on the endothelial cells. These features suggest that PECAM-targeted drug delivery will be robust and can be used for either prophylaxis or therapies, i.e., drug delivery to either normal or pathologically altered vasculature (see Figure 4 below).

Initial studies revealed that although endothelial cells in culture avidly bind anti-PECAM, the antibodies poorly accumulate in the animal lungs after intravenous administration. In addition, endothelial cells did not internalize anti-PECAM. However, anti-PECAM conjugation (e.g., by streptavidin cross-linker) provides multimeric anti-PECAM complexes that are readily internalized by endothelial cells (see Figure 1) and accumulate in the animal lungs after vascular administration (see Figure 2) (Muzykantov et al., 1999). Size of the anti-PECAM conjugates controls their intracellular uptake: neither monomolecular anti-PECAM, nor large (>500 nm diameter) conjugates undergo internalization. Conjugates within 100-300 nm size range are readily internalized by endothelium (Wiewrodt et al., 2002).

This paradigm, facilitation of the internalization and pulmonary targeting by forming 100-300 nm anti-PECAM conjugates, has been employed to achieve an effective, intracellular targeting of diverse cargoes to endothelial cells in cell cultures, perfused rat lungs and in intact animals. For example, an active reporter enzyme, beta-galactosidase conjugated to anti-PECAM, has been shown to accumulate intracellularly in the pulmonary

endothelium as soon as 10 min after intravenous injection in mice and pigs (Scherpereel et al, 2002). Anti-PECAM/DNA conjugates provide specific transfection of endothelial cells in culture (Wiewrodt et al., 2002) and in animals (Li et al., 2000).

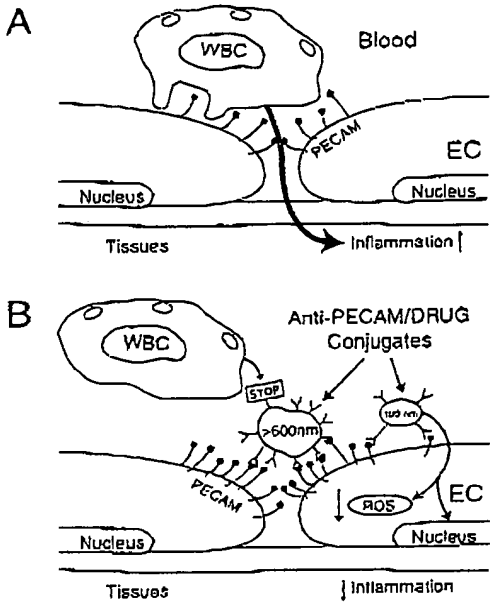

Figure 4. Immunotargeting to PECAM-1 (see explanations in the text). WBC – white blood cells, EC – endothelial cells; ROS – reactive oxygen species. Panel A. PECAM-1 constitutively expressed by endothelium, predominantly in the intercellular contacts, supports WBC transmigration and facilitates inflammation in the tissues. Panel B: Large (>600 nm diameter) anti-PECAM conjugates poorly internalize in the endothelial cells, whereas smaller counterparts (100-300 nm diameter) enter the cells and can be used for intracellular delivery of genetic materials and anti-oxidant enzymes to detoxify ROS. In addition, anti-PECAM conjugates may suppress inflammation via blocking WNC transmigration.

Glucose oxidase (GOX, an enzyme generating H_2O_2 from glucose) has been conjugated to anti-PECAM and tested in cell cultures and mice. In both models, the conjugate demonstrated highly effective and specific binding to the endothelium, internalization, generation of H_2O_2 inside the cell, and acute oxidative stress that lead to a severe edematous lung injury in mice (Christofidou et al, 2002). This result, targeting of active ROS-generating enzymes *in vivo*, confirms that vascular immunotargeting to constitutive endothelial antigens provides biologically significant effects. In addition, anti-PECAM/GOX can be used to model oxidative stress and vascular pulmonary injury in laboratory animals (Christofidou et al., 2002). Theoretically, targeting of GOX or other ROS-generating enzymes to the antigens presented

on tumor cells or in the tumor vasculature promises a complementary approach for tumor eradication.

Potential therapeutic applications of anti-PECAM targeting are similar to that of ACE: intracellular delivery of enzymes and genetic materials. For example, anti-PECAM-conjugated catalase binds to endothelial cells, enters the cells, protects them against oxidative stress and accumulates in animal lungs after intravenous injection (Muzykantov et al., 1999). The pilot studies showed that anti-PECAM/catalase affords significant protective effects in animal models of oxidative stress including acute lung transplantation injury, likely due to attenuation of the ischemia/reperfusion syndrome. However, pending applications anti-PECAM and anti-ACE strategies are not identical: the former can be used for both prophylaxis and therapy (including pathological conditions that suppress ACE targeting). In addition, blocking of PECAM-mediated leukocyte extravasation may suppress inflammation, thus providing an important secondary therapeutic benefit in certain groups of patients.

Inter-Cellular Adhesion Molecule, ICAM-1

ICAM-1 (CD54) is an Ig superfamily surface glycoprotein with a short cytoplasmic domain, a transmembrane domain, and a large extracellular domain (Diamond et al., 1990). It is constitutively expressed by the endothelium at a relatively high surface density ($2x10^4$-$2x10^5$ copies per cell). Other cell types also express ICAM (e.g., alveolar epithelial cells, macrophages); however, the major fraction of ICAM accessible to the bloodstream is exposed by the luminal surface of endothelium. Pathological stimuli, such as ROS, cytokines, abnormal shear stress, and hypoxia, stimulate expression of ICAM by the endothelium and thus elevate the ICAM surface density in pulmonary vasculature.

As a counter-receptor for integrins, ICAM supports leukocytes' firm adhesion to the endothelium and facilitates inflammation. In addition, ICAM serves as a natural ligand for certain viruses and serves as a signaling molecule; yet the exact mechanisms, specificity, and significance of this signaling in different cell types remains to be more fully understood. Antibodies (including humanized murine mAbs) directed against ICAM-1 (anti-ICAM) suppress leukocyte adhesion, thus producing anti-inflammatory effects in animal models and clinical pathological settings associated with vascular injury, such as acute inflammation, ischemia/reperfusion, and oxidative stress (DeMeester et al., 1996).

Accumulating data indicate that: (a) ^{125}I-anti-ICAM binds to endothelium *in vivo*; (b) accumulates in highly vascularized organs (first of all, lungs); and (c) inflammation facilitates both processes (Panes et al., 1995; Villanueva et al., 1998). Therefore, ICAM-1 is a good candidate determinant for drug targeting to normal and inflammation-engaged endothelia, especially in the lungs. The published data on *in vivo* effects of anti-ICAM conjugates are rather limited. However, in the model of isolated perfused rat lung, catalase conjugated with anti-ICAM provides a protective effect similar to that of catalase conjugate with anti-ACE (Atochina et al., 1998).

The data on anti-ICAM internalization is fragmentary and controversial. Several laboratories reported that the epithelium and leukocytes internalize ICAM ligands, while data from other groups implies that other cell types internalize anti-ICAM rather poorly (Almenar-Queralt et al., 1995; Mastrobatista et al., 1999). Our preliminary data indicate that the endothelium poorly internalizes monomeric anti-ICAM, as well as large (>500 nm diameter) anti-ICAM conjugates, while 100-300 nm conjugates are internalized via a mechanism that resembles anti-PECAM conjugates uptake. The pilot results show that large anti-ICAM/tissue-type plasminogen activator conjugates accumulate in the lungs, are retained on the endothelial surface, and facilitate fibrinolysis in the pulmonary vasculature.

Collectively, the available data reveal important unique features of ICAM as a target and suggest that anti-ICAM can be used for targeted delivery of diverse drugs to the endothelium:

i) ICAM-1 is presented on the endothelial surface at high density and easily accessible to drugs circulating in the bloodstream, while its level in blood (i.e., plasma ICAM) is low, thus permitting a robust and specific binding of anti-ICAM conjugates to endothelium in the vasculature.

ii) Endothelial ICAM-1 is stably expressed and, in contrast with other constitutive endothelial surface antigens, its surface density is increased in pathological conditions. Thus, one could anticipate an effective or even facilitated targeting to pathologically altered endothelium.

iii) Inhibition of ICAM-1 function by targeting (blockage of leukocyte adhesion to endothelium) may suppress inflammation, a benefit for treatment of vascular oxidative and thrombotic stress. The anti-inflammatory effect of anti-ICAM conjugates may be even more potent.

iv) Depending on the size of anti-ICAM conjugates, they may target active cargoes either to the endothelial surface (e.g., plasminogen activators) or to the intracellular compartment (e.g., catalase).

Anti-ICAM is reported to deliver liposomes and other cargoes to the vascular endothelium (Atochina et al., 1998; Bloeman et al., 1998; Villanueva et al., 1998). The preliminary data from our laboratory strongly suggest that anti-ICAM can deliver fibrinolytics to the surface of the pulmonary endothelium for facilitation of anti-thrombotic potential of the pulmonary vasculature in cases of high risk of thromboembolism.

Endothelial selectins

Two types of selectin molecules are found in the endothelium: E- and P-selectins. Both types serve as counterparts for specific sugars on the white blood cells and support the first phase of their adhesion ("rolling") to the endothelial cells in the sites of inflammation. Selectin molecules are not normally expressed on the luminal surface in the vasculature. Resting endothelial cells store P-selectin in the intracellular vesicles (i.e., Weibel-Palade bodies) and do not synthesize E-selectin. However, pathological mediators stimulate rapid mobilization of P-selectin to the endothelial surface, as well as synthesis and surface expression of E-selectin.

These determinants theoretically may provide a good means for specific targeting of drugs to the inflammation-engaged endothelium. Radiolabeled E-selectin antibodies accumulate in the sites of vascular inflammation (Keelan et al., 1994). Most likely, the targeting might produce a secondary anti-inflammatory effect due to blocking of leukocyte rolling, yet this hypothesis has yet to be proven in animal models. Importantly, E-selectin is an internalizable molecule (Kujpers et al., 1994) that permits intracellular uptake of the targeted conjugates liposomes and viruses (Spragg et al., 1997; Harrari et al., 1999). A relatively low surface density of selectins on the surface of activated endothelial cells, the transient nature of their expression, and presence of P-selectin on platelets has generated concerns about the robustness, applicability, and specificity of the targeting. However, targeting selectins represents an exciting and promising area that is discussed in some detail in Chapters 8 and 20.

Thrombomodulin, caveoli-associated antigens and other determinants

Thrombomodulin (TM) is a transmembrane endothelial glycoprotein that binds thrombin, and, in concert with protein C, changes thrombin

substrate specificity and thus converts it from a pro-coagulant enzyme into an anticoagulant one that inactivates coagulation factors (Esmon, 1989). Endothelial cells in the pulmonary capillaries are rich in thrombomodulin, which apparently plays an important role in anti-thrombotic protection of the lung vasculature that collects blood clots from the venous circulation. Steve Kennel and co-authors explored monoclonal antibodies against thrombomodulin as an affinity carrier for targeting the pulmonary endothelium and reported that anti-TM diverse cargoes, including radiolabels and liposomes, accumulated in the murine lungs (Maruyama et al., 1990). However, targeting compromises thrombomodulin function and might predispose or even provoke thrombosis (Christofidou et al., 2002). Therefore, clinical application of anti-TM is questionable due to obvious safety concerns related to the pulmonary thrombosis, yet this delivery system has an important application as an animal model.

Caveoli represent a specialized domain in the endothelial plasma membrane involved in intracellular and trans-cellular trafficking, cellular signaling, and sensing of flow. The pulmonary endothelium is rich in caveoli; several caveoli-associated antigens are being explored as potential determinants for the targeting and intracellular delivery of therapeutic cargoes. One such determinant is gp60, or endothelial albumin-binding protein. Malik and co-workers found that binding of ligands to this protein induces internalization and may permit intracellular delivery (Tirrupati et al., 1997). Schnitzer and co-workers have produced a monoclonal antibody against another caveoli-associated antigen, gp90, (McIntosh et al., 2002) that binds to endothelial cells in the rat pulmonary vasculature, enters the cells, and provide intracellular and trans-cellular targeting of radiolabels and tracer cargoes (see Chapter 6 by Carver and Schnitzer). Theoretically, targeting caveoli represents an exciting avenue that may extend targeting towards sub-endothelial layers, pending identification of functions and human counterparts of these proteins, as well as validation of the targeting and effects of therapeutic agents in animal models.

Recently, a novel endothelial surface determinant, a transmembrane glycoprotein gp85, has been found in a unique area of pulmonary capillary endothelium, namely, the avesicular zone – a thin part of an endothelial cell that lacks major organelles and, together with the thin Type I alveolar epithelium and the basal membrane, separates alveolar and vascular compartments (Ghitescu et al., 1999). Interestingly, a monoclonal antibody against this determinant displays very selective and robust accumulation in the pulmonary vasculature in rats without significant internalization (Murciano et al., 2001). Therefore, this determinant might be considered as a target for the pulmonary delivery of anti-thrombotic agents. However, the function, human counterpart, therapeutic and side effects of targeting gp85

remain to be characterized in order to evaluate its applicability for the targeting pulmonary endothelium.

There are several other endothelial surface determinants identified in the pulmonary vasculature, including Thy-1.1 antigen (Danilov et al., 2001). However, their functions and the effects of targeting remain to be addressed in order to evaluate whether they can be used for targeting.

CONCLUSIONS AND PERSPECTIVES

The pulmonary endothelium represents an important therapeutic target. Many endothelial determinants that are potentially useful for drug delivery have been identified recently using diverse methodologies, including administration of radiolabeled monoclonal antibodies and phage display library *in vivo* (Danilov et al., 1991; Muzykantov et al., 1996; Rayotte et al., 1997; Muzykantov, 1998; Danilov et al., 2001; McIntosh et al., 2002). Some determinants, such as ACE, ICAM and PECAM, can be used for targeting either normal (i.e., prophylaxis) or pathologically altered (i.e., therapy) endothelium, whereas selectins permit specific recognition of pathological endothelium. Targeting caveoli provides an exciting avenue for intracellular and transcellular targeting in the pulmonary vasculature, whereas modulation of the antibody-drug conjugate size provides a novel paradigm for control of intracellular uptake and trafficking of the cargoes. Targeting of active reporter and therapeutic cargoes, including enzymes, genes and viruses, has been recently achieved in intact animals, thus providing a good basis for pre-clinical evaluation of the strategy. Many targeting systems promise unique advantages for delivery of specific therapeutic agents in diverse clinical settings and, therefore, must be carefully tested in terms of the robustness, effectiveness, specificity and effects of the targeting (including the effects mediated by intervention in the functions of target determinants). Based on the current progress rate in the field, it would be not too unrealistic to expect that immunotargeting to the pulmonary endothelium will be in the clinical studies during this decade.

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GLOSSARY

Alveolar capillaries: Small (5-25 micron diameter) blood vessels surrounding pulmonary alveoli that are involved in gas exchange and metabolization of vasoactive peptides. The alveolar endothelium is a privileged vascular target that represents roughly 30% of total endothelial surface in the human body.

Angiotensin-converting enzyme (ACE): A transmembrane endothelial glycoprotein, an ectopeptidase that controls activity of vasoactive peptides angiotensin and bradykinin. ACE is a candidate determinant for targeting pulmonary endothelium.

Angiotensin I and II: An octapeptide Ang II, produced by ACE from a decapeptide Ang I, induces vasoconstriction and exerts pro-oxidant activity.

Bradykinin: A vasoactive peptide that elevates endothelial permeability and stimulates endothelial production of NO.

Catalase: An enzyme that detoxifies H_2O_2 and could be used as a drug. One of the candidate therapeutic cargoes for vascular immunotargeting to protect the endothelium and treat an acute pulmonary oxidative stress.

Caveoli: A specialized cholesterol-rich domain in the endothelial plasma membrane that forms characteristic flask-shape invaginations and serves as

sensing, signaling and endocytotic compartments. Caveoli represent a unique and highly promising target for endothelial drug delivery.

Intercellular adhesion molecule-1 (ICAM-1): A transmembrane endothelial glycoprotein that facilitates leukocytes' adhesion. ICAM-1 is a candidate determinant for targeting pulmonary and systemic endothelium

Localization Ratio: The ratio between the uptake of a drug or a reporter molecule in an organ of interest and its blood level. Comparison of this parameter in different organs and tissues helps to characterize tissue selectivity of the targeting.

Nitric Oxide, NO: A small gaseous molecule produced by endothelial cells that diffuses in blood and vascular tissues and exerts strong vasorelaxing and anti-thrombotic properties.

Platelet-Endothelial Adhesion Molecule-1 (PECAM-1): A transmembrane endothelial glycoprotein that facilitates leukocytes' transmigration from blood to tissues. PECAM-1 is a candidate determinant for targeting pulmonary and systemic endothelium.

Reactive oxygen species (ROS): Pro-oxidant molecules such as superoxide anion and H_2O_2 that are produced in the vasculature normally at low levels. Massive overproduction of ROS under pathological conditions causes oxidative stress and tissue injury.

Selectins: Selectins P and E are surface adhesion molecules involved in leukocyte adhesion to the endothelium, transiently expressed on the surface of pathologically altered endothelial cells, for example, in inflammation foci. The selectins represent candidate determinants for selective targeting pathological endothelium.

Thrombomodulin (TM): A transmembrane endothelial glycoprotein that indirectly suppresses coagulation and thus helps to prevent intravascular thrombosis. The pulmonary endothelium is enriched in TM that can be useful for experimental targeting; yet clinical applications of TM antibodies are questionable due to potential thrombosis.

Vascular immunotargeting: A strategy for delivery of therapeutics to vascular cells (e.g., endothelium) using antibodies or their fragments as affinity carriers.

Endothelial Endocytic Pathways: Gates for Vascular Drug Delivery

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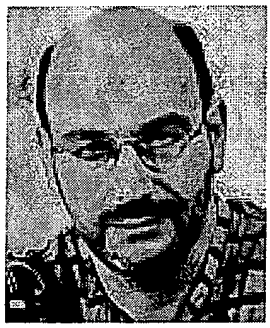

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Abstract: Vascular endothelium plays strategic roles in many drug delivery paradigms, both as an important therapeutic target itself and as a barrier for reaching tissues beyond the vascular wall. Diverse means are being developed to improve vascular drug delivery including stealth liposomes and polymer carriers. Affinity carriers including antibodies or peptides that specifically bind to endothelial surface determinants, either constitutive or pathological, enhance targeting of drugs to endothelial cells (EC) in diverse vascular areas. In many cases, binding to endothelial surface determinants facilitates internalization of the drug/carrier complex. There are several main endocytic pathways in EC, including clathrin- and caveoli-mediated endocytosis, phagocytosis and macropinocytosis (these two are less characteristic of generic EC) and the recently described Cell Adhesion Molecule (CAM)-mediated endocytosis. The latter may be of interest for intracellular drug delivery to EC involved in inflammation or thrombosis. The metabolism and effects of internalized drugs largely depend on the routes of intracellular trafficking, which may lead to degrading lysosomal compartments or other organelles, recycling to the plasma membrane or transcytosis to the basal surface of endothelium. The latter route, characteristic of caveoli-mediated endocytosis, may serve for trans-endothelial drug delivery. Pericellular trafficking, which can be enhanced under pathological conditions or by auxiliary agents, represents an alternative for transcytosis. Endothelial surface determinants involved in endocytosis, mechanisms of the latter and trafficking pathways, as well as specific characteristics of EC in different vascular areas, are discussed in detail in the context of modern paradigms of vascular drug delivery.

Keywords: Drug delivery, Vascular endothelium, Endocytosis, Transcytosis, Paracellular transport, Intracellular trafficking.

1. INTRODUCTION. VASCULAR ENDOTHELIUM: A BARRIER AND TARGET FOR DRUG DELIVERY

The inner surface of blood vessels is lined with endothelial cells (EC) strategically positioned to control vascular physiology. EC control numerous vital functions and represent an extremely important target and barrier for drug delivery.

For instance, numerous vasoactive factors secreted by EC and including (yet most likely not limited to) NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF), suppress contractility of the vascular smooth muscle cells and therefore control vascular tone [1, 2].

EC help to control thrombosis [1]. For example, in concert with plasma protein C, endothelial transmembrane glycoprotein thrombomodulin converts thrombin into an anti-coagulant enzyme [3]. Among other anti-thrombotic factors, EC secrete NO and prostacyclin, which suppress platelet aggregation, as well as urokinase and tissue type plasminogen activators that dissolve blood clots *via* generation of the fibrin-degrading protease plasmin.

The endothelium is also involved in inflammation, a process that is often intertwined with thrombosis [4, 5]. Under normal circumstances, EC provide very limited, if any, support for activities of pro-inflammatory cells (e.g., white blood cells, WBC). However, pathological mediators, including cytokines, reactive oxygen species (ROS), growth factors and abnormal shear stress, induce endothelial secretion of chemoattractants and exposure of adhesion molecules leading to leukocyte attraction, adhesion and transmigration [6-9].

Furthermore, EC play an important role in normal and pathological vascular redox mechanisms [10]. They produce ROS (superoxide anion O_2^- and H_2O_2) *via* enzymatic pathways that can be further activated by pathological mediators [11]. ROS apparently play an important role in cellular signaling. However, ROS overproduction by EC or by activated WBC leads to vascular oxidant stress, inactivation of NO by O_2^-, lipid peroxidation and tissue injury [12].

EC function is modulated by exposure to dynamic environmental factors such as blood flow shear stress, aggressive inflammatory cells and compounds in the circulation, including lipids, proteases, oxidants and xenobiotics. Disruption of normal endothelial function can exacerbate the pathology of a number of diseases including atherosclerosis, hypertension, thrombosis, diabetes, acute lung injury and sepsis. Improper EC function can also lead to defects in angiogenesis, which in turn, could either inhibit vascularization during wound repair resulting in tissue necrosis or cause inappropriate blood vessel formation, which can lead to tumor vascularization. Therefore, EC

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represent an important potential target for delivery of therapeutic agents including, but not limited to, antioxidants to protect against oxidative stress, anticoagulants and fibrinolytics to manage thrombotic stress, NO donors to reduce vessel tone and blood pressure, and anti-inflammatory agents to suppress vascular inflammation.

The total surface area of vascular endothelium in the human body approaches the size of a tennis court (~ 240 m^2), making it a large and highly accessible target for drugs circulating in the bloodstream. Most therapeutic agents, however, have no specific affinity to this target. Therefore, only a small fraction of injected drugs produces a therapeutic effect in endothelium, whereas the major fraction is handled as a waste product, or worse, produces deleterious side effects. Furthermore, binding to target cells is necessary, but not sufficient, for effective action of many drugs, which require internalization and proper sub-cellular addressing. Thus, it is a key issue in the design of drug delivery vehicles to create carriers that are targeted to EC and also are trafficked by the target cells to destinations where they can have maximal efficacy. Recognizing this problem, more and more groups are focusing their research efforts on the design of novel strategies for targeted delivery of therapeutics to EC [13-26].

With other layers of the blood vessel wall, the endothelium forms a barrier for delivery of drugs to extravascular targets, such as tumors, brain and myocardium. Understanding the mechanisms involved in trans-cellular and pericellular endothelial transport may permit more effective delivery of these drugs. Among other parameters, endothelial uptake and transcytosis depend on the size of a transported agent. Barrier function of endothelium is especially restrictive for large therapeutic molecules (e.g., proteins or genetic materials) and drug delivery systems that employ carrier nanoparticles, liposomes, or high molecular mass polymers.

Therefore, the endothelium is an extremely important tissue in the context of vascular drug delivery, either as a target itself for therapeutic interventions by a delivered drug or as a barrier on route to peripheral tissues [27, 28]. Targeted delivery of drugs to endothelium and control of their internalization, sub-cellular addressing and transendothelial transport are critically important components for the rational design of safe, effective and specific therapies. The goal of this article is to review these issues in the context of drug delivery systems designed for targeting of drugs to and beyond EC.

2. A BRIEF OVERVIEW OF VASCULAR DRUG DELIVERY SYSTEMS

Most known drugs lack specific binding and uptake by target organs. On the other hand, many drugs undergo rapid inactivation in the body by aggressive components of blood and special cellular detoxifying systems. In addition, some drugs including therapeutic enzymes (e.g., proteases), have specific inhibitors in the blood. Also, drugs can be eliminated through a number of mechanisms, including clearance from the bloodstream by non-specific targets (e.g., red blood cells and hepatocytes) and specific clearance systems (e.g., reticuloendothelial system and renal filtration). This often dictates that administration of large doses is required for efficacy, also increasing potentially dangerous side effects.

Elimination and inactivation of pharmacological agents is decreased when a therapeutic "cargo" is loaded into or conjugated with natural or artificial carriers increasing bioavailability of a drug (e.g., liposomes, polymer nanoparticles, lipoproteins, blood cells or proteins) [27, 29-37]. Coating of drugs or their carriers by activated polyethylene glycol, PEG, forms an aqueous shell masking against natural protective mechanisms including macrophages, complement and immune cells, a strategy sometimes referred to as "stealth" technology [38-40]. PEG-coating markedly prolongs circulation of drugs and reduces side effects associated with the immune response and systemic activation of host defense including complement and leukocytes.

In addition, many delivery systems such as liposomes or conjugation with carrier lipoproteins, facilitate intracellular uptake of drugs [41]. The mechanisms of this phenomenon are complex and depend on specific cell type and carrier (see below). However, there are several mechanisms employed by currently available drug carriers to facilitate intracellular uptake.

First, drugs conjugated with ligands of cellular receptors can be internalized *via* vesicle-mediated pathways. Endocytosed carriers usually follow the natural itinerary their receptors would normally traverse to the point of dissociation or degradation in intracellular compartments such as lysosomes. For example, cells expressing transferrin receptor internalize drugs conjugated or genetically fused with transferrin *via* clathrin-mediated endocytosis [42]. Second, fusion-competent liposomes can be designed to enter the cells *via* endocytic pathways, leading to delivery and fusion with intracellular membrane organelles, or to fuse with lipids in the plasma membrane, thus injecting their content directly into the cytosol. In an attempt to control the extent and specificity of cytosolic delivery, liposome carriers have been designed that are destabilized after internalization at acidic pH in endocytic compartments prior to release of a drug or fusion with cell membranes [43]. Third, antibodies and their derivatives with multivalent binding sites facilitate cellular entry of liposomes [44-48]. Fourth, a more recent approach to cytosol delivery has been to couple proteins and sub-micron particles with the TAT-peptide from HIV virus, which in some instances can enter cells in an energy independent manner, although they may require endocytosis in other cases [49, 50]. This and some other charged peptides enriched with lysine and/or arginine promiscuously permeate cell membranes and enable cytosolic delivery to diverse cell types *in vitro* and *in vivo* [51, 52].

Therefore, diverse natural carriers (e.g., lipoproteins or transferrin) or synthetic carriers (e.g., liposomes) can facilitate vascular drug delivery and intracellular uptake. In the next section we consider how these means can be employed in the context of specific drug targeting to EC.

3. ENDOTHELIAL SURFACE DETERMINANTS: POTENTIAL TARGETS FOR DRUG DELIVERY

Stealth liposomes, protein carriers and other delivery systems prolong the circulation time of drugs and may enhance their cellular uptake, but do not confer an affinity to

EC. Unless drugs are coupled to high-affinity carriers, the circulation removes drugs and their derivatives rapidly, even after local infusion *via* vascular catheters. In these instances, the major fraction of injected materials ends up in the liver and not in EC. Devices allowing a transient cessation of blood flow in the site of catheter placement have been designed in order to attain a high local concentration and a more effective prolonged interaction of the infused material with endothelium, but blood flow interruption may lead to ischemia and vascular injury.

Viable means for effective, rapid, and safe targeting of therapeutic molecules to EC are beginning to emerge to address this important and persisting biomedical problem. In order to facilitate targeting, cargoes or their carriers can be conjugated (chemically or genetically) with affinity moieties that bind to EC. Antibodies directed against endothelial surface determinants and small antigen-binding fragments of these antibodies represent one of the most useful classes of affinity carriers for targeted drug delivery to EC. Indeed, coupling drugs with carrier antibodies permits targeted delivery to EC (vascular immunotargeting) [19, 53].

Immunostaining of tissues, *in vivo* selection of peptide ligands using phage display and tracing labeled antibodies in animals have been used to identify several EC antigens that potentially can be used as targets [14, 15, 20, 21, 54-57]. However, no universal or ideal carrier suits all therapeutic needs. Specific therapeutic goals require different secondary effects mediated by binding to EC, drug targeting to different sub-populations of EC (e.g., resting *vs.* inflammation-engaged EC), and to diverse cellular compartments. Also, targeted delivery of antioxidants or NO-donors to normal or resting EC can be useful for either prophylaxis or therapies. On the other hand, specific recognition and drug delivery to abnormally activated or pathologically altered EC might permit more specific means for treatment of such maladies as localized tumor growth and inflammation. Table **1** shows some endothelial determinants useful for experimental vascular targeting to EC, which may have therapeutic potential.

Several surface determinants are potentially useful for targeting either normal and/or pathologically altered EC. For example, antibodies to thrombomodulin (TM), a constitutively expressed EC antigen, can be used for targeting of diverse cargoes to the endothelium [58]. Unfortunately, TM is functionally "untouchable" for therapies, because its inhibition by anti-TM may cause thrombosis [59]. However, TM antibodies are being successfully utilized for delivery of reporter or toxic compounds to the pulmonary EC in animal models [60].

Angiotensin-converting enzyme (ACE) is a transmembrane glycoprotein expressed on the endothelial luminal surface, which converts Ang I into Ang II to induce vasoconstricting, pro-oxidant and pro-inflammatory activities [61-63]. Pulmonary vasculature is enriched in

Table 1. Endothelial Determinants: Selected Candidate Targets for Drug Delivery

Target	Function and Localization	Targeting Advantages	Potential Problems
ACE	Peptidase, converts Ang I into Ang II and cleaves bradykinin. ACE enriched in the lung capillaries.	Selective targeting to lung EC. Intracellular delivery. Vasodilating and anti-inflammatory effects of ACE inhibition.	Inflammation suppresses targeting. ACE inhibition may be dangerous.
TM	Binds thrombin and converts it into an anti-coagulant enzyme. Enriched in the lungs.	Intracellular delivery to EC useful for modeling of lung injury in animals.	Inflammation suppresses targeting. Thrombosis due to TM inhibition.
PECAM	Facilitates transmigration of leukocytes. Stably expressed in EC borders.	Intracellular delivery of anti-PECAM conjugate may also suppress inflammation	PECAM-signaling and side effects are not understood
ICAM	Mediates leukocyte adhesion to EC. Stably expressed by EC, and up-regulated by pathological agents.	Similar to PECAM, but inflammation enhances targeting.	Similar to PECAM
E-selectin	Supports leukocytes adhesion. Expressed only on altered EC.	Intracellular targeting to EC in inflammation	Targeting is not robust. Transient expression.
P-selectin	Similar to E-selectin	Similar to E-selectin	Similar to above. Targeting platelets
gp90	Function unknown. Localized in EC cavoli.	Transendothelial targeting.	Human analogue and side effects are not known.
gp85	Function unknown. EC avesicular zone in alveolar capillaries.	Targeting to the EC surface	Similar to above
gp60	Albumin-binding protein in EC caveoli	Transendothelial targeting	Side effects and specificity of targeting are not known

EC – endothelial cells, ACE – angiotensin-converting enzyme, TM - thrombomodulin, PECAM - platelet-endothelial adhesion molecule, ICAM - intercellular adhesion molecule; gp – glycoproteins.

ACE: nearly 100% of EC in the alveolar capillaries are ACE-positive *vs* <15% ACE-positive EC in the extra-pulmonary capillaries [64]. Radiolabeled anti-ACE accumulates in the pulmonary vasculature after intravascular and intraperitoneal injections in rats, cats, primates and humans [14, 17]. Diverse reporter compounds and drugs conjugated with anti-ACE accumulate selectively in the lungs after intravenous injection in rats [14, 17, 65]. Recently, anti-ACE has been used successfully for re-targeting of viruses to pulmonary EC in rats [25, 26].

ACE also inactivates bradykinin, a peptide stimulating NO production, although Ang II may stimulate NO production by EC [66]. Some anti-ACE antibodies block its active site and/or facilitate ACE shedding from the endothelium by specific secretases regulated by metalloproteases [67-69]. However, other ACE antibodies enable ACE to retain its function. Therefore, using ACE antibodies directed to different epitopes enables targeting strategies to be developed that either retain or inhibit ACE activity, enhancing flexibility and therapeutic applicability of the strategy. ACE inhibition may be beneficial in conditions associated with vascular oxidant stress, ischemia and inflammation.

Pro-inflammatory agents (e.g., ROS) suppress endothelial expression of ACE [70, 71] which may inhibit therapeutic targeting. However, anti-ACE is a good candidate for targeting to the pulmonary endothelium for a prophylactic use; it does not cause acute harmful reactions in animals and humans [17]. EC internalize anti-ACE that may deliver drugs intracellularly [65]. Anti-ACE-conjugated antioxidant enzymes such as catalase, accumulates in rat lungs *in vivo* [72] and protect perfused rat lungs against H_2O_2 [73].

Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM, CD31) is a pan-endothelial transmembrane Ig superfamily glycoprotein (m.w. 130 kD), predominantly localized in the sites of cellular contacts in the endothelial monolayer [74]. Platelets and WBC also express PECAM, but at levels that are orders of magnitude lower than EC. PECAM is abundant in EC, which express millions of anti-PECAM binding sites [75]. In addition, PECAM is a stable EC antigen: cytokines and ROS do not down regulate its expression and surface density on the endothelium. This promises a robust PECAM-targeted drug delivery to either normal or pathologically altered vasculature, for either prophylaxis or therapies.

PECAM is involved in the cellular recognition, adhesion, signaling, and trans-endothelial migration of leukocytes [74]. Adhesion and transmigration of leukocytes is involved in pathogenesis of many disease conditions including inflammation, sepsis, atherosclerosis, acute lung injury and diabetes [76-78]. Animal studies showed that blocking PECAM by administration of anti-PECAM suppresses inflammation and protects organs against leukocyte-mediated oxidant stress [77, 79]. Therefore, anti-PECAM targeting may provide secondary benefits for management of inflammation, perhaps by attenuation of leukocyte transmigration.

EC bind anti-PECAM without internalization, but anti-PECAM conjugation (e.g., by streptavidin) provides multimeric anti-PECAM complexes that are readily internalized by endothelium and accumulate in animal lungs in perfusion or after IV administration [64, 75]. An active reporter enzyme, beta-galactosidase, conjugated to anti-PECAM has been shown to accumulate intracellularly in the pulmonary endothelium as soon as 10 min after IV injection in mice and pigs [80, 81].

InterCellular Adhesion Molecule-1 (ICAM-1, CD54) is another Ig superfamily surface glycoprotein with a short cytoplasmic domain, transmembrane domain and large extracellular domain [6, 82-84]. It is normally expressed by EC at relatively high surface density ($2x10^4$-$2x10^5$ surface copies per cell). Some other cell types also express ICAM-1 (e.g., alveolar epithelial cells, macrophages). However, the major fraction of blood-accessible ICAM-1 is exposed on the luminal surface of EC. Several laboratories demonstrated robust and specific binding of radiolabeled ICAM antibodies and anti-ICAM conjugates to vascular endothelium after intravenous administration in diverse laboratory animals [64, 73, 85-87].

Pathological stimuli, such as ROS, cytokines, abnormal shear stress and hypoxia stimulate surface expression of ICAM-1 by EC *via* signaling mechanisms involving activation of MAP kinases and nuclear translocation of NF-kappaB [88, 89]. ROS and cytokines elevate the ICAM-1 surface density in pulmonary EC [90, 91]. Therefore, in contrast with some other constitutive endothelial determinants (e.g., TM and ACE), immunotargeting to ICAM-1 is not suppressed, but instead is markedly facilitated in inflammation and other pathological conditions [85-87, 92-96].

ICAM-1, a counter-receptor for integrins on WBC, supports their firm adhesion to EC and thus contributes to inflammation [97-100]. In addition, ICAM-1 serves as a natural ligand for certain viruses [101]. ICAM-1 may also serve as a signaling molecule, yet the exact mechanisms, specificity and significance of this signaling in different cell types remains to be more fully elucidated. Antibodies (including humanized murine mAbs) directed against ICAM-1 suppress leukocytes adhesion to EC, thus producing anti-inflammatory effects in animal models and clinical pathological settings associated with vascular injury, such as acute inflammation, ischemia/reperfusion and oxidant stress [102-106]. Blocking of endothelial ICAM-1 by targeting may inhibit leukocyte adhesion to EC and thus suppress inflammation, a benefit for treatment of vascular oxidant and thrombotic stress. The anti-inflammatory effect of anti-ICAM conjugates may be even more potent due to their potentially higher affinity/valency and down regulation of surface ICAM-1 *via* internalization (see below).

Antibodies directed against constitutive cell adhesion molecules PECAM and ICAM described above do not discriminate between EC in different vascular areas, thus providing "pan-endothelial targeting". Anti-PECAM and anti-ICAM directed conjugates accumulate preferentially in the lungs after intravenous administration (due to the fact that pulmonary vasculature represents about 30% of the total vascular surface in the body and receives 100% cardiac first pass venous blood output), whereas injecting *via* catheters inserted in a conduit artery facilitates local delivery in the downstream vascular area [64, 81]. Local delivery may also

be enhanced by surface endothelial determinants enriched in particular vascular areas or in focal pathological processes.

For example, glycoprotein gp85 identified by Ghitescu [107] is predominantly localized in the thin part of EC body that separates alveolar and vascular compartments and lacks main organelles ("avesicular zone"); gp85 monoclonal antibodies accumulate in rat pulmonary vasculature without internalization [108]. On the other hand, animal studies showed that the pulmonary endothelium in rats contains surface determinants localized to cholesterol-enriched plasma membrane microdomains, including caveoli. Ligands of determinants localized to caveoli such as gp60 and gp90 also accumulate in the pulmonary vasculature after intravenous injection in rats, enter EC and traverse endothelial barrier [57]. The functions and human counterparts of these endothelial determinants are not known and, thus their potential utility as targets for drug delivery is not clear. However, caveoli-localized determinants might provide an exciting opportunity for trans-endothelial drug delivery (see Section 6).

Endothelium in the cerebral vasculature represents a specially important and difficult target. Recent animal studies showed that carrier antibodies and peptides directed to several surface determinants relatively enriched in EC in the brain, including receptors for transferrin, insulin, putrescin and some growth factors, permit delivery of the reporter compounds and genes into the brain [109-111]. Importantly, some of these endothelial receptors apparently permit transendothelial drug delivery into the brain tissue and neurons (see in Section 6).

EC exposed to inflammatory mediators and abnormal shear stress show cell surface expression of P-selectin, normally stored intracellularly and mobilized rapidly to the surface, and E-selectin, which is newly synthesized by activated EC [7]. Therefore, selectins are transiently exposed on the surface of stressed EC [112, 113]. Experiments in cell cultures and limited animal studies show that selectins may permit targeting of drugs to cytokine-activated endothelium [16, 114-118].

EC in solid tumors also represent a specially important and challenging target for delivery of agents designed to visualize tumors, inhibit angiogenesis, or eradicate malignant cells [119-121]. Tumor vasculature is characterized by numerous morphological abnormalities [122, 123]. EC in tumor vessels expose abnormal determinants including selectins (see above), integrins, apoptosis markers and receptors for growth factors [124-128]. Targeting these determinants in tumors might be useful to accomplish two goals: i) inflict damage in the tumor vasculature leading to thrombosis, infarction and starvation of malignant cells [18, 129] and, ii) delivery of anti-tumor agents to the proper malignant cells using, for example, PEG-immunoliposomes or polymers loaded with taxol or doxorubicin [130, 131]. The latter approach involves permeation of the endothelial barrier, mostly *via* paracellular pathways (see Section 6). EC seem to internalize protein carriers modified with peptides containing RGD sequence to provide recognition of integrins over-expressed on tumor endothelium [132].

In summary, affinity carriers directed to diverse determinants presented on surface of normal or pathologically altered EC, permit targeted delivery of reporters and, perhaps, therapeutic cargoes to the vascular endothelium. In addition to this delivery function, affinity carriers may provide additional means to control rate of internalization and sub-cellular or trans-cellular traffic. This subject will be considered in more detail in the following sections.

4. MECHANISMS OF ENDOTHELIAL ENDOCYTOSIS

Endocytosis is a complex and delicately coordinated process, which involves an extensive cellular machinery to mediate the formation of membrane transport vesicles to enable the internalization of extracellular material (reviewed in [133-135]). In endothelium, which is strategically positioned at the interface between blood vessels and interstitial milieu, endocytosis has a prime role in the maintenance of body homeostasis by regulating transendothelial gradients and the transport of macromolecules (reviewed by [136, 137]). Depending upon the ultimate fate of the internalized vesicles, endocytic events in EC have been categorized as either endocytosis, i.e., the uptake of fluids, biomolecules and other ligands that sort to endothelial processing pathways, or transcytosis that reflects transport across the cells to the subendothelial space [138-140]. EC employ multiple mechanisms for vesicle-mediated membrane transport (see Fig. **1** and Table **2**). The mechanism of endocytosis is frequently dictated by membrane receptors used by extracellular ligands to bind to the plasma membrane as a prerequisite to internalization.

Caveolar-mediated uptake plays an important role in endothelial transport functions (reviewed by [141-144]). Caveolar-mediated endocytosis is preferentially inhibited by chelators of cholesterol (e.g., filipin or cyclodextrin) and is mediated by interactions of the coat protein caveolin with cell signaling and cytoskeletal molecules. Internalization *via* caveoli is involved in the uptake of glycolipids, GPI-anchored proteins, and chemokines [145-148], dynamic recycling of brain microvascular EC membranes [149], constitutive turnover of TM [150], potocytosis of folate and other solutes [151], and can participate in the regulation of the vascular permeability [152]. Importantly, caveolar-mediated endocytosis serves as an entry point for transcytosis of many compounds through the endothelial monolayer from the bloodstream to sub-endothelial tissues (see Section 6).

Clathrin-mediated endocytosis, which is the predominant form of receptor-mediated endocytosis in most cell types, is less prominent in EC than caveolar-mediated endocytosis. Nonetheless, numerous cases of clathrin-mediated endocytosis have been reported among EC, particularly, in EC in hepatic sinusoids, which participate in clathrin-mediated internalization of IgG immune complexes *via* Fc receptors [153]. Mannose-terminated glycoproteins or lactosilated albumin particles are internalized through mannose or galactose-specific receptors [154, 155]. Also, colloidal gold coated with mannan, albumin, or thrombospondin aggregates on coated pits and is taken up by EC in sinusoids in liver [156-159]. This is also the case for chondroitin sulphate proteoglycan attached to gold particles,

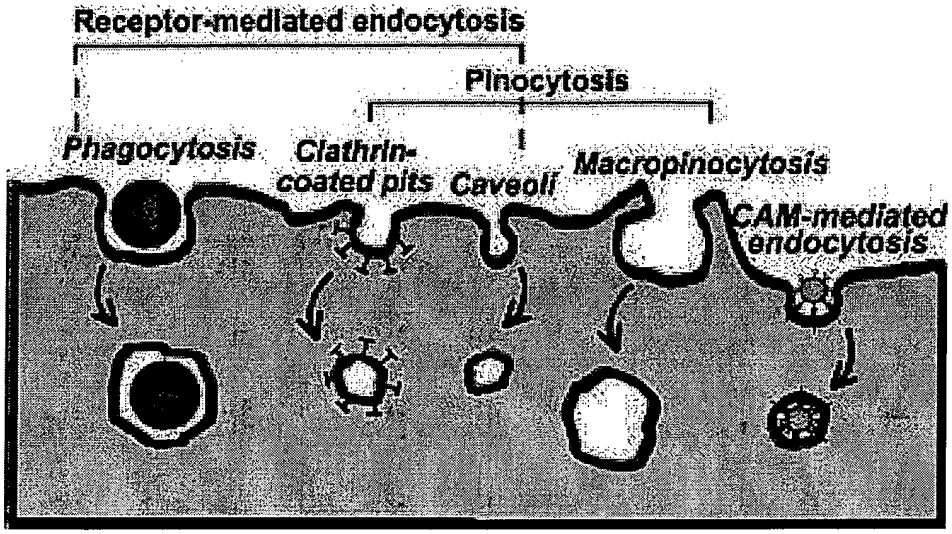

Fig. (1). *Endocytic pathways.* Endocytosis accounts for the internalization of extracellular material into cells, mediated by formation of transport vesicles derived from the plasma membrane. The terms "phagocytosis" and "pinocytosis" refer to the uptake of large particulate ligands and extracellular medium, respectively. In addition, macromolecular ligands can bind to specific receptors at the plasma membrane, triggering their internalization by what is known as "receptor-mediated endocytosis". This term contrasts with "macropinocytosis", which consists in the non-adsorptive bulk uptake of extracellular fluids. Cell adhesion molecule (CAM)-mediated endocytosis is stimulated by clustering Ig superfamily CAMs. "Clathrin-" and "caveoli"-mediated pathways are ubiquitous endocytic mechanisms, whereas "phagocytosis" and "macropinocytosis" are most typically presented by specialized cells (e.g., macrophages, dendritic cells). The five different pathways depicted have been found to some extent in EC, yet "caveoli"-mediated endocytosis seems to be the most active process.

Table 2. Endocytic Pathways and Inhibitors

Internalization pathway	Inhibitor	Molecular target
Clathrin-mediated	Potasium depletion	Clathrin dissociation
Clathrin-mediated	MDC	Protein interaction at latices
Clathrin-mediated	Amantadine	Vesicle budding
Caveoli	Genistein	Tyrosine kinases
Caveoli	Filipin	Cholesterol sequestration
Caveoli	Cyclodextrin	Cholesterol extraction
Multiple	Dynamin K44A, PH*	Dynamin
Macropinocytosis/Phagocytosis	Cytochalasin	Actin filaments
Multiple	Latrunculin	Monomeric actin
Macropinocytosis	Amiloride	Na^+/H^+ exchanger
Macropinocytosis	BIM-1, H7, staurosporin	Protein kinase C
EGF/BCR receptor uptake	Radicicol	Src kinase
Rho dependent uptake	Y27632	ROCK
Macropinocytosis/Phagocytosis	Wortmannin	PI3 kinase

MDC – Monodansyl cadaverine. Dynamin K44A – dominant negative dynamin affected at the ATPase site. Dynamin PH* - dominant negative dynamin affected at the plecktrin homology domain. BIM-1 – Bisindolylmaleimide-1. H7 - 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine. ROCK – Rho dependent kinase. PI 3 kinase – phosphatidyl inositol 3 kinase.

which bind to hyaluronic acid/chondroitin sulphate receptor at the plasma membrane and undergo internalization by coated pits in rat liver EC [160].

The endothelium in many organs (i.e. spleen, bone marrow, thymus, or brain) has been shown to internalize ligands through clathrin-mediated endocytosis, as is the case for acetoacetylated or acetylated LDL [161], gold-coated LDL or insulin [162], and transferrin [163, 164]. Also, EC likely internalize lipoprotein lipase (LPL) by clathrin-related mechanisms. LPL synthesized and secreted by EC is retained on the abluminal side, being further transported across the cell to the apical space [165], where it provides intravascular hydrolysis of triacylglycerol-rich lipoproteins [166] and also contributes to LDL-holoparticle turnover and selective uptake of LDL-associated lipids [167]. Internalization of LPL within the cell might occur as a consequence of its interaction with heparan sulphate proteoglycans during turnover or recycling of the latter [168]. For instance, LPL stimulates endocytosis of LDL upon binding of membrane-associated heparan sulphate [169] and also enhances binding, uptake and degradation of glycated LDL in a manner independent of LDL receptor and LDL-receptor related protein [170]. Additionally, LPL has been found to bind with high affinity to the glycoprotein gp330 in microvascular EC [171, 172]. Although the localization of gp330 in EC remains unclear, this is typically concentrated to clathrin-coated areas in epithelial cells, indicating that LPL internalization might be mediated by a clathrin-related pathway [173]. However, lipoprotein interactions with the syndecan family of endothelial proteoglycans also can lead to internalization *via* a clusterization-induced pathway distinct from coated pits endocytosis [174].

Clathrin-coated pits also mediate constitutive internalization of plasma membrane proteins, as is the case for E- and P-selectins, two inducible endothelial adhesion molecules whose recycling and/or surface expression is regulated by clathrin-mediated uptake [112, 175]. The inducible expression of selectins has been used as a means to enable intracellular delivery to activated EC. For instance, anti-E-selectin targeted liposomes or conjugates have been found to internalize *via* clathrin-coated pits within EC, to enable intracellular delivery of anti-inflammatory drugs [176, 177].

Another membrane internalization process represented in EC, although to a low extent, is phagocytosis (reviewed in [178, 179]). Phagocytosis, which is typically displayed by macrophages and antigen presenting cells, accounts for the internalization of large (> 1μm) particulate ligands [180]. This requires initial binding of the particle to specific receptors (i.e. scavenger receptor, C3R, FcγR, etc), with subsequent activation of a battery of signaling cascades (i.e. phosphatidyl inositol 3 kinase and Rho family GTPases, among others) (reviewed by [181]). In many instances, these events drive a major redistribution of the actin cytoskeleton. As a consequence of the cortical actin polymerization, either pseudopods or large invaginations form at the cell surface, where the plasma membrane surrounds the particulate ligand in a zipper-like mechanism [182], finally resulting in a cup-shape invagination. This is the case of vascular EC, which have been reported to uptake aged red blood cells and apoptotic cells with phosphatidylserine exposed in the outer layer of the cell surface, upon binding of these to the lectin-like oxidized LDL receptor 1 (LOX-1) [183]. *Neisseria meningitidis* induces the formation of cellular protrusions *via* activation of Rho and Cdc42 and recruitment of ezrin and moesin to the cortical membrane, which mediate bacterial entrance in EC [184].

In contrast to caveoli-mediated, clathrin-mediated endocytosis and phagocytosis, macropinocytosis [185, 186] is generally considered a non-receptor mediated process, where cells uptake large volumes of extracellular fluids and solutes (classical macropinosomes are > 1μm in diameter), during a mechanism that involves active formation of membrane ruffles and protrusions at the plasma membrane [187-189]. This is reminiscent of phagocytosis, where macropinocytosis requires large re-arrangements of the actin cytoskeleton, with involvement of protein kinases C and Rho family small GTPases as central signal transduction players [190-194]. In general, however, macropinocytosis is constitutive in highly specialized cells (i.e. macrophages and dendritic cells) and can be induced by growth factors in epithelial cells [195-197], but does not seem to be a principal endocytic pathway in EC. Nevertheless, it has recently been found that human immunodeficiency virus (HIV) can access brain microvascular EC by a macropinocytic mechanism, apparently involving endothelial ICAM-1 [198].

However, neither phagocytosis, macropinocytosis nor classical clathrin-mediated endocytosis seem to be principal endocytic pathways in vascular endothelium. In fact, coated-pits are much less abundant than non-coated vesicles in capillary endothelium in lung [199]. For example, hormones like insulin or gonadotropin [200, 201], long chain fatty acids such as oleate [202], or ligands for TM [203, 204], have been described to enter vascular EC *via* coated pits as a minor fraction, whereas caveoli account for the internalization of the main pool.

Interestingly, two adhesion molecules constitutively expressed in EC, ICAM-1 and PECAM-1, present the capability to drive internalization of small multivalent ligands, although neither these molecules nor their monomeric ligands (i.e. antibodies) have been found to undergo endocytic uptake in endothelium [75, 87, 205, 206]. However, major group human rhinovirus and respiratory syncytial virus use ICAM-1 as a receptor during cell invasion, although the mechanism of cell uptake remains uncertain [207, 208]. Also, PECAM-1 is required for binding of malaria infected red blood cells to EC in culture [209] and homophilic PECAM-1 interaction displays a signaling role during recognition and phagocytic ingestion of apoptotic leukocytes by macrophages [210].

This offers a pathway for intracellular delivery of therapeutic cargoes, by using small multimeric conjugates of ICAM-1 and PECAM-1 antibodies. These have been recently shown to enter EC by a non-classical endocytic mechanism, CAM-mediated endocytosis, which is distinct from classical clathrin- or caveolar-mediated uptake as well as from phagocytic and macropinocytic processes [206]. In particular, internalization of anti-ICAM-1 or anti-PECAM-1 conjugates was dependent on antigen clustering, and a critical parameter for internalization was conjugate size (100

- 300 nm in diameter). CAM-mediated endocytosis also required Rho kinase- and protein kinase C- mediated rearrangements of the actin cytoskeleton [206].

Anti-PECAM/DNA conjugates provide specific transfection of EC in culture [205] and in mice [22]. Furthermore, anti-PECAM-conjugated glucose oxidase generates H_2O_2 in cultured EC intracellularly in cell culture [211] and induces acute oxidative endothelial injury in murine lungs after IV injection [212]. Recent studies indicate that anti-CAM/catalase conjugates bind to and enter EC, which protects endothelium against oxidant stress in cell cultures [75, 213] and in animals [214].

5. INTRACELLULAR TRAFFICKING AND FATE OF INTERNALIZED MATERIALS

The endocytic pathways reviewed above can be used for the intracellular or transcellular delivery of therapeutic cargoes in EC. Therefore, control of the cellular processes driving the internalization of site-specific therapeutics is important in order to achieve their optimal effects. Intracellular trafficking of a drug can be dictated by its entry pathway; hence determining its final destination and degradation rate. For instance, internalized membranes and/or contents can be selected for recycling to the cell surface, transported through the cell body and exocytosed by transcytosis, sorted to other sub-cellular destinations such as Golgi, trans-Golgi network (TGN) or endoplasmic reticulum (ER), or most typically processed for delivery to specialized intracellular compartments responsible for degradation of internalized materials (see Fig. **2** and Table **3**).

In this context, there are three main systems accounting for protein degradation in mammalian cells. Cytosolic proteins are degraded by proteosomes or calpains, whereas lysosomal proteases digest proteins within the lumen of endocytic vesicles. Calpains are cysteine proteases localized

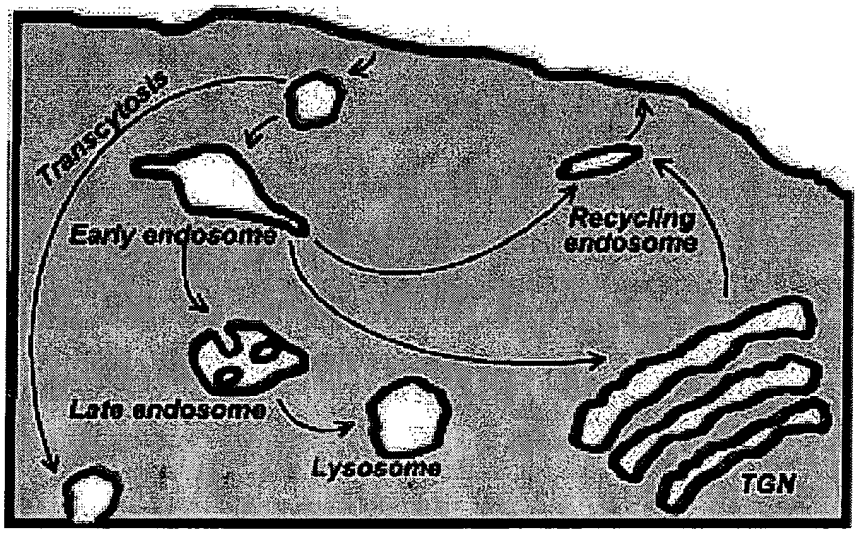

Fig. (2). *Intracellular trafficking.* Endocytic vesicles, containing membrane receptors and their respective ligands or other contents, can be selected for sorting to different sub-cellular compartments. For instance, these can recycle to the cell surface by "recycling endosomes" (e.g., directly or through Trans Golgi Network (TGN) compartments) or transported through the cell body and exocytosed to the abluminal space by "transcytosis". Internalized material can also be processed for delivery to endocytic compartments for degradation (e.g., by trafficking through "early endosomes", "late endosomes" and "lysosomes"), or sorted to other sub-cellular destinations such as Golgi or the Endoplasmic Reticulum.

Table 3. Intracellular Trafficking and Inhibitors

Trafficking pathway	Inhibitor	Molecular target
Trans Golgi network (TGN) trafficking	Brefeldin	TGN/ER-Arf1
Receptor recycling	Chlorpromazine	Recycling
Trafficking to lysosomes and recycling	Monensin	Na^+/H^+ exchange at the endosome
Lysosomal degradation	Ammonium chloride, chloroquine	Vesicle acidification
Lysosomal degradation	Bafilomycin	Vacuolar H^+-ATPase
Endosome-lysosome trafficking	Nocodazole, colchicine	Microtubular network

to the cytoplasm and nucleus of all cell types studied (reviewed by [215]). They undergo activation by Ca^{2+} and phospholipids, which promote targeting of calpains to membranes. In this situation, calpains can exert proteolytic activity, resulting in modification of enzyme [216] and receptor function [217, 218]. Also, cytoskeleton-related proteins can undergo modification by calpain activity, as is the case for calpain-dependent disruption of integrin/ cytoskeleton interactions [216, 219-221].

Another cytosolic degradation system, the proteosome, is also primarily located in the cytosol of all eukaryotic cells. Proteosomes are multisubunit complexes (i.e., 20S, 26S, the immunoproteosome, and the hybrid proteosome), which contain a common core and different additional regulatory subunits (reviewed by [222]). For instance, cytokine-dependent immunoproteosome activation has been related to the production of peptides that will act as ligands of MHC-I [223]. On the other hand, activation of the hybrid proteosome (depending on ATP but not on ubiquitin) generates peptides that are targeted to ER lumen [224]. The function of the proteosome 26S containing subunits that confer this complex the ability to selectively recognize, bind and cleave ubiquitin-tagged proteins has been extensively studied [222]. The process of protein degradation by the ubiquitin system requires initial attachment of ubiquitin residues to the protein substrate by ubiquitin-protein ligases, which recognize particular post-translational modifications in the target proteins. Subsequently, the ubiquitin-tagged proteins will be degraded by the 26S proteosome complex, concomitantly with the release of reusable ubiquitin residues.

However, most typically lysosomes are responsible for proteolysis of internalized material, since most endocytic traffic merges with lysosomal compartments. The endolysosomal system contains a series of differentiated vesicles including early endosomes, late endosomes and lysosomes. Early endosomes are the first compartment where sorting of materials to recycling, degradation pathways or other subcellular organelles is decided [225-229]. In this context, H^+-ATPases and Na^+, K^+-ATPases favor acidification of the endosomal lumen, whereas Na^+/H^+-exchangers help to generate a positive potential that inhibits proton influx [230]. These combined actions regulate the pH at the early endosome lumen, which becomes to be around 6.3-6.5. This mildly acidic pH favors separation of some ligands from their receptors, being the latter typically recycled to the plasma membrane, whereas ligands tend to traffic to late endosomes, as is the case of LDL-R or transferrin-R (reviewed by [231]). Alternatively, the receptor/ligand complex can also be recycled as a unit, or the complex can be entirely targeted to degradation [232].

When sorted to lysosomal compartments, materials traffic to late endosomes. Here, the vacuolar-ATPase pumps H^+ to the vesicle lumen [233], being responsible for the further acidification of this compartment to a pH around 5-5.5 [234]. Regulation of pH also depends on Cl^- channels directed inwardly to counteract the positive membrane potential created by the vacuolar-ATPase activity [235]. The compartments continue to acidify to very low pH (pH 4.8) and materials traffic to lysosomes, the terminal compartment in the degradation pathway. Lysosomes appear as electron dense bodies, where the entrapped materials can be then rapidly degraded by lysosomal acidic proteases [236].

Lysosomal hydrolysis is regulated by proton ATPases and cysteine transporters, as mentioned above, and also by lysosome-associated membrane proteins, the main protein constituent of endolysosomal membranes (reviewed by [237]). The latter are heavily glycosylated transmembrane proteins, referred to as LAMPs, which protect lysosomes from the action of degradative enzymes [238]. Hydrolysis at lysosomal compartments is carried out by proteases (i.e. cathepsins), including both exopeptidases (cysteine and serine proteases) and endopeptidases (cystein and aspartic proteases) [237, 239]. These proteases are extensively glycosylated, which facilitates their trafficking to lysosomes and helps confer resistance to low pH [236].

An example of a typical endocytic pathway, which delivers the internalized materials to the endolysosomal system, is clathrin-mediated internalization, although this can also sort the internalized contents to recycling or transcytotic pathways, after rapid removal of the clathrin coat from the membrane of the vesicles. For instance, transferrin-R or P-selectin can transit from the cell surface to endosomes and TGN, following by trafficking back to the EC surface [163, 175]. However, rapid degradation of P-selectin in lysosomes can also occur as a consequence of its frequent passage through endosomal compartments [175].

Also, a variety of ligands such as IgG immune complexes, glycoproteins, ferritin, thrombospondin, or colloidal gold coated with albumin, insulin, LDL or chondroitin sulphate proteoglycans are transported within minutes to lysosomal compartments in hepatic endothelial sinusoids [153, 154, 157-160, 162, 240]. Moreover, site-specific delivery vehicles targeted to endothelium through E-selectin (i.e. anti-E-selectin liposomes and conjugates) traffic rapidly to multivesicular bodies and other acidic compartments [176, 177], which could considerably reduce the half-life of potential cargoes. In epithelial cells, IgA is internalized by clathrin-mediated endocytosis and subsequently transcytosed from the basolateral to the apical plasma membrane [241]. Whether this pathway also operates in EC remains to be determined, however, some ligands internalized by EC through caveolae are transcytosed (see Section 6).

Phagocytosis typically results in lysosomal delivery. Phagosomes rapidly undergo uncoating of the actin-based machinery after internalization, with recycling of the plasma membrane proteins and degradation of receptors and content by sequential fusion with endosomes and lysosomes [109, 242-244]. Similarly, during macropinocytosis, the internalized contents can be recycled to the cell surface, but more typically are processed by delivery to the endosome-lysosome system. The first case can occur by tubulation of macropinosomes with fission into small vesicular intermediates [245], whereas classically, macropinosomes mature to acidic degradation vesicles by sequential interactions with pre-existing compartments [185, 246].

Ligands internalized by caveolar-mediated endocytosis can be sorted to several intracellular compartments using a variety of sorting pathways, with an interesting capability of

avoiding lysosomal compartments. For instance, cholera toxin traffic through early and late endosomes [145, 146], and SV40 is sorted from early endosomes to Golgi and finally ER [247, 248]. Some ligands, such as folate, can be delivered to the cytoplasm during a process called potocytosis, where folate is transported across the plasma membrane while folate receptor remains associated with caveolae at the plasma membrane [249]. Others, such as alkaline phosphatase, bradykinin, acetylcholine and endothelin, are returned to the cell surface after their internalization [147, 250-252]. Therefore, internalization *via* caveoli tends to favor sorting to sub-cellular compartments other than lysosomes and thus may be useful to avoid rapid degradation pathways. Also caveolar-mediated uptake provides a rapid mechanism for membrane turnover in EC, where plasma membrane components traffic through tubular endosomes and TGN in a constant influx-efflux cycle [149].

As mentioned above, EC can also use a ligand-induced endocytic pathway known as CAM-mediated endocytosis [206]. This internalization pathway delivers materials to lysosomal compartments with unusually slow kinetics (around 3 h) [253]. Conjugation of catalase to anti-ICAM-1 or anti-PECAM-1 antibodies permits intracellular delivery of the active enzyme to EC, and provides anti-oxidant protection for a relatively prolonged window time due to the slow kinetics of delivery to lysosomes [214, 253].

The protective effect of anti-CAM delivered catalase can be further prolonged either in the presence of drugs acting on the microtubular-network (i.e. nocodazole) [253], which is involved in traffic to lysosomes [254], or using weak bases (i.e. chloroquine), which impair acid-dependent activation of degradation enzymes in these compartments [253].

6. TRAVERSING ENDOTHELIAL BARRIERS *VIA* TRANSCYTOSIS AND PARACELLULAR TRANSPORT

In cases when a drug must be delivered to malignant cells in tumors, across the Blood Brain Barrier (BBB) or cardiomyocytes in the heart and other extravascular targets, endothelium represents a barrier that must be traversed for the therapeutic effect. In theory, drugs and macromolecules can extravasate *via* two pathways: transcytosis (i.e., endocytosis on the apical surface followed by traffic through the EC body to the basolateral surface) or diffusion *via* intercellular junctions (paracellular transport). The role and contribution of these two pathways in migration of macromolecules from the bloodstream into tissues is a subject of intense research and ongoing discussions. The theoretical concept of endothelial transcytosis of macromolecules involving dynamic or stable trans-cellular vesicular channels has been postulated by Majno and Palade in the 1960s [255, 256]. However, even today the mechanisms, regulation, significance and therapeutic utility of this pathway remain very much elusive [257].

Numerous studies from several groups including experiments in perfused organs and intact animals strongly indicate that transcytosis represents a significant route across the vascular endothelial barrier [57, 258-263]. When considering transcytosis for the purposes of drug delivery, ligands are endocytosed by the apical (lumenal) aspect of the endothelial plasma membrane, followed by transport through a series of intracellular compartments and delivery to the basolateral (ablumenal) plasma membrane where they are secreted.

An alternate model for transcytosis is through the formation of an organelle, called the vesiculo-vacuolar organelle (VVO) that may form dynamic or stable channels through cells [264]. Both morphological and functional evidence including studies of transendothelial transfer of labeled compounds in animal models show the presence of a VVO in EC [57, 264]. However, relatively little is known about mechanisms of VVO formation and how it can be manipulated pharmacologically. For example, histamine and VEGF seem to stimulate transport *via* VVO formation [264], but the specificity of this functional modulation is difficult to interpret, because these vasoactive agents also facilitate pericellular transport (see below).

Endothelial transcytosis often originates from the caveolar endocytotic pathway, where plasma solutes and macromolecules are taken up from the bloodstream in bulk by fluid phase adsorption (glycogen, dextran, ferritin, etc) or by binding to specific receptors (LDL, ceruloplasmin, etc) and reach the abluminal space [138, 258, 260, 265-267]. Plasma proteins such as albumin, TM, chorionic gonadotropin, insulin and growth factors can be transported to the abluminal surface of the EC *via* caveoli-mediated transcytosis [200, 201, 266, 268]. The levels of plasma lipoproteins and cholesterol homeostasis are believed to be maintained largely by endothelium in arteries which, due to continuous caveolar uptake, serves to reconstitute the levels of cholesterol in the vessel wall and permits the transit and subsequent accessibility of cholesterol-carrying lipoproteins to sub-endothelial layers of the vessel wall and peripheral tissues [269, 270].

Several studies indicated that interaction of a protein ligand leading to receptor clustering in caveoli and activation of specific signaling pathways plays a critical role in initiation of transcytosis [260, 261]. For example, caveolar clustering of endothelial albumin binding protein gp60 has been shown to increase transendothelial permeability by mediation of phosphorylation events including signaling through the Src family of tyrosine kinases [271]. Interestingly, chemical modifications of caveolar ligands, such as albumin nitration (that may take place in oxidant stress and inflammation) even further stimulate transcytosis [272].

Caveolar transcytosis pathways are envisioned as means for transcellular delivery of therapeutics, which could be achieved by targeting caveolae-located receptors. For example, active reporter compounds conjugated with antibodies directed against specific antigen (gp90) localized in pulmonary endothelial caveolae undergo transendothelial and transepithelial transport through pulmonary endothelium from the blood stream to the alveolar space after injection *in vivo* [57]. Although the function of gp90 and potential effects of its inhibition by targeting are unknown, it is tempting to speculate that caveolar targeting will permit more effective extravascular drug delivery.

In this context, transcytosis through the BBB represents a specific interest. The paracellular pathways (see below) in

this vascular area are relatively restricted, yet the BBB endothelium is not all that impermeable and yet can be traversed *via* aplethora of agents (for reviews see [273, 274]). From a biological standpoint, this can be illustrated by the fact that *Escherichia coli* invades the central nervous system by transmigration through brain microvascular EC using an actin- and microtubule-dependent phagocytic mechanism, although this pathway was inactive in systemic EC [275]. From a drug delivery standpoint, it is important that ligands of insulin, putrescine and transferrin receptors are expressed on the BBB endothelium. Also, antibodies and high-affinity binding peptides defined using a BBB endothelial phage-display library show an encouraging ability to accumulate in the brain tissue [32, 276]. Moreover, proteins and genetic "drugs" or reporter compounds conjugated with these affinity carriers display their activities in the brain tissue after systemic administration, suggesting transmission across the BBB [110, 111, 277].

In addition to transcytosis mechanisms, the density and permeability between cells of the endothelial monolayer varies between different organs; in this respect, endothelium can be roughly categorized into three types. The first type is fenestrated endothelium, which is localized to organs of the reticuloendothelial system, including hepatic and spleenic sinuses and bone marrow. Fenestrae is Latin for "windows" and refers to the relatively large pores between the fenestrated EC. These pores can be up to several microns in diameter and thus permit the relatively unhindered extravasation of blood components, including red and WBC in these tissues. A second type is continuous endothelium, which is present in most of other organs such as cardiac, pulmonary and mesenterial blood vessels. Continuous endothelium is relatively tighter than fenestrated endothelium and has fewer pores with 100 nm diameters. Although this can permit passage of macromolecules to tissues, in most cases, filtration through the continuous endothelium is restricted to small molecules and solutes such as glucose and small hormones. The third type is microvascular endothelium, which is the most restricted in permeability. In particular, brain microvascular EC form an extremely dense monolayer, resulting in an extremely tight BBB, which lacks significant permeability for plasma proteins and possesses specific enzymatic systems (glycoprotein P, e.g.) to facilitate reverse transport of molecules from the brain tissue to the circulation [274]. Clearly, due to these differences in vascular permeability, BBB endothelium represents the most formidable biological barrier for the extravascular drug delivery, while fenestrated endothelium does not restrict extravasation of even large carriers such as liposomes, polymer particles and viruses [278].

However, in many organs (e.g., liver) and types of vasculature (e.g., venules) the mainstream of transendothelial transport for nano-scale drug delivery vehicles such as liposomes and polymer carriers follows paracellular pathways. Paracellular endothelial permeability is controlled by proteins which form the tight junction complex localized to contact sites between EC. Proteins in the claudin family form the basis for regulating paracellular permeability [279, 280]. Claudins are not a simple barrier, per se, since many claudins form paracellular channels that enable selective ion permeability. Also, different tissues express different claudins, suggesting at least some regulation at the level of transcription. Recently, a definitive role for claudin-5 in regulating the BBB was demonstrated since substrates less than 1 kD molecular mass were freely permeable across the BBB in claudin-5 deficient mice [281]. Moreover, claudin function and paracellular permeability is also regulated by associated transmembrane proteins (occludin, junction adhesion molecule (JAM), connexins, VE-cadherin) and peripheral proteins, which tether it to the actin cytoskeleton such as ZO-1, ZO-2, ZO-3 and PATJ [282-284]. For instance, HGF/SF treated vascular EC show increased permeability due to ZO-1 phosphorylation, with no effect on claudin expression levels, suggesting a change in barrier function due to disruption of the ZO-1/claudin-1 complex [285]. The ability of VEGF and thrombin to increase endothelial permeability may also be due to a comparable upstream effect on signaling cascades that alter tight junction organization. Hormone activation and cross talk between these different classes of junction proteins and the regulation of claudin function is just beginning to be elucidated and is an active area of research.

The array of different tight junction associated proteins suggests many avenues for pharmacological manipulations of vascular permeability (see [286]). Studies using clostridium toxin, which binds to the extracellular domains of claudin-3 and claudin-4 and disrupts intestinal epithelial barrier function provide a basis for the notion that tight junction interfaces can be disrupted by targeting claudins. Thus, it seems plausible that another class of small molecules or perhaps claudin antibodies might provide an alternative strategy for preferential and transient disruption of EC tight junctions. In particular, the selective change in BBB paracellular permeability exhibited by claudin-5 deficient mice suggests that specific modulation of claudin-5 may have potential as a method to temporarily alter BBB permeability [281]. Conversely, blockade of junction proteins may help increase endothelial barrier function, as indicated above, where anti-ICAM-1 conjugates appear to inhibit leukocyte transmigration.

Many pathological mediators and conditions including hypoxia, hyperoxia, histamine, VEGF, thrombin, hyperthermia, abnormal shear stress, cytokines and ROS elevate vascular permeability [287-291]. However, some compounds (e.g., adenosine) tend to restrict endothelial permeability in cell cultures [292]. Some permeability-enhancing agents (histamine, VEGF) apparently stimulate both transcytosis [122] and pericellular transport [291, 293]. Vasoactive agents inducing paracellular permea-bility cause elevation of cytosolic Ca^{2+} in EC [294], activation of kinases leading to myosin light chain phosphorylation and subsequent endothelial contraction and reorganization of adhesion contacts [77, 288, 295, 296].

Vascular cells, including the endothelium itself, can regulate vascular permeability *via* the action of diverse vasoactive substances, which regulate porosity of endothelial monolayer and blood pressure (the higher intravascular pressure, the more effective extravascular transport). Interestingly, one modality of a vasoactive agent (e.g., its effect on blood pressure) frequently is balanced by its other

modality (effect on permeability). For example, endothelial ACE converts Ang I into Ang II, which induces vasoconstriction, yet ACE also inactivates bradykinin and substance P [61], peptides that increase vascular permeability. EC produce a potent vasodilating agent NO that may reduce vascular permeability at low levels [297].

Due to activities of substance P, thrombin, ROS, VEGF, bradykinin and other kinins endothelial permeability is usually enhanced under pathological conditions including inflammation, which can exacerbate edema [257]. Both these factors and morphological alterations (fenestrations) enhance permeability of tumor vasculature and cause tumor vascular leakiness [123, 290, 298]. This phenomenon is being actively exploited in design of liposomal and polymer carrier-based strategies for drug delivery to tumors (enhanced permeability and retention effect, EPR) [299].

The role of transcytosis *vs* paracellular transport of particular compounds under normal and pathological conditions is controversial and is the subject of continuing discussions. For example, although liposomes can undergo endothelial transcytosis in cell cultures, they likely leave the vasculature *via* pericellular pathways in animals [278]. Experiments in perfused organs showed that albumin extravasation is independent on temperature, suggesting that this process represents a passive diffusion *via* intercellular pores rather than *via* energy-dependent endocytotic pathways [137]. Mice genetically deficient in caveolin, a critical component of caveolar endocytotic pathway, do not show overt abnormalities of tissue transport of plasma components [300, 301]. However, both transcellular and pericellular pathways can and should be employed for rational design of modern drug delivery systems. In theory, pericellular pathways may be more useful for extravasation of drugs in tumors and inflammation foci, whereas endothelial transcytosis might serve as a pathway for drug delivery through the BBB.

CONCLUSION AND PERSPECTIVES

Recent years have produced a wealth of knowledge of molecular mechanisms regulating pathways for endothelial entry, intracellular traffic, degradation or recycling and transcytosis of diverse materials, including therapeutic agents. Optimal endothelial and transendothelial drug delivery promises substantial improvements of effectiveness and safety of therapeutic strategies for treatment of cardiovascular (e.g., atherosclerosis, hypertension, ischemia-reperfusion syndrome), pulmonary (e.g., acute lung injury, hyperoxia, pulmonary hypertension), and metabolic diseases (e.g., diabetes), inflammation (e.g., sepsis and endotoxemia) and tumor growth. One key to effective treatment of these distinct pathologies is the ability to control the rates and precise destinations of endothelial drug delivery. There is a plethora of potential avenues for achieving this challenging and exciting goal.

Optimal selection of endothelial surface determinants and design of drug delivery system molecular characteristics such as valency of binding to endothelium, charge and size will be likely used to control the uptake and fate of drugs. Utilization of cross-linkers sensitive to minute changes in pH or specific cellular proteases (e.g., lysosomal cathepsin G) can be employed for the release of active drugs at selected stages of their intracellular traffic. Conjugation of drugs or drug vehicles with membrane fusion and chaperone peptides will help to achieve yet even more precise sub-cellular localization in organelles such as mitochondrion, nucleus or peroxysomes.

In some cases, therapeutic needs might require methods to decelerate or avoid endothelial internalization such as in case of delivery of anti-thrombotic and other agents, which need to exert their activity in the vascular lumen. Therefore, using non-internalizable determinants (e.g., monomeric anti-ICAM-1) or deceleration of natural endocytotic pathways (e.g., by using vehicles with diameter exceeding the limit of internalization) could be used for this goal. Transcytotic and pericellular transport mechanisms could be employed in the cases when a drug should be delivered beyond the EC.

In summary, binding, entry and traffic of drugs into and through EC represent central paradigms of many drug delivery strategies serving diverse therapeutic goals. Future progress in understanding the control of these processes will provide more effective and safe therapeutic means.

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ABBREVIATIONS

ACE	=	Angiotensin-Converting Enzyme
Ang I	=	Angiotensin 1
Ang II	=	Angiotensin 2
BBB	=	Blood Brain Barrier
CAM	=	Cellular Adhesion Molecule
EDHF	=	Endothelium-Derived Hyperpolarizing Factor
ER	=	Endoplasmic Reticulum
EGF	=	Epithelial Growth Factor
EC	=	Endothelial Cell(s)
GPI	=	Glycosyl Phosphatidyl Inositol
HGF	=	Human Growth Factor
ICAM-1	=	Intercellular Adhesion Molecule-1
JAM	=	Junction Adhesion Molecule
LOX-1	=	Lectin-like Oxidized LDL receptor-1
LPL	=	Lipoprotein Lipase
LAMP	=	Lysosomal Associated Membrane Protein 1
LDL	=	Low Density Lipoprotein
LDL-R	=	Low Density Lipoprotein Receptor
NO	=	Nitric Oxide

PEG	=	Polyethylene Glycol
PECAM	=	Platelet EC Adhesion Molecule-1
ROS	=	Reactive Oxygen Species
ROCK	=	Rho-dependent Kinase
RBC	=	Red Blood Cell
Transferrin-R	=	Transferrin Receptor
TM	=	Thrombomodulin
TGN	=	Trans-Golgi Network
VVO	=	Vesiculo-Vacuolar Organelle
VEGF	=	Vascular Endothelial Growth Factor
WBC	=	White Blood Cells

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Lung uptake of antibodies to endothelial antigens: key determinants of vascular immunotargeting

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Danilov, Sergei M., Vitaly D. Gavrilyuk, Folker E. Franke, Katarina Pauls, David W. Harshaw, Timothy D. McDonald, David J. Miletich, and Vladimir R. Muzykantov. Lung uptake of antibodies to endothelial antigens: key determinants of vascular immunotargeting. *Am J Physiol Lung Cell Mol Physiol* 280: L1335–L1347, 2001.—Vascular immunotargeting is a mean for a site-selective delivery of drugs and genes to endothelium. In this study, we compared recognition of pulmonary and systemic vessels in rats by candidate carrier monoclonal antibodies (MAbs) to endothelial antigens platelet endothelial cell adhesion molecule (PECAM)-1 (CD31), intercellular adhesion molecule (ICAM)-1 (CD54), Thy-1.1 (CD90.1), angiotensin-converting enzyme (ACE; CD143), and OX-43. Tissue immunostaining showed that endothelial cells were Thy-1.1 positive in capillaries but negative in large vessels. In the lung, anti-ACE MAb provided a positive staining in 100% capillaries vs. 5–20% capillaries in other organs. Other MAbs did not discriminate between pulmonary and systemic vessels. We determined tissue uptake after infusion of 1 μg of ^{125}I-labeled MAbs in isolated perfused lungs (IPL) or intravenously in intact rats. Uptake in IPL attained 46% of the injected dose (ID) of anti-Thy-1.1 and 20–25% ID of anti-ACE, anti-ICAM-1, and anti-OX-43 (vs. 0.5% ID of control IgG). However, after systemic injection at this dose, only anti-ACE MAb 9B9 displayed selective pulmonary uptake (16 vs. 1% ID/g in other organs). Anti-OX-43 displayed low pulmonary (0.5% ID/g) but significant splenic and cardiac uptake (7 and 2% ID/g). Anti-Thy-1.1 and anti-ICAM-1 displayed moderate pulmonary (4 and 6% ID/g, respectively) and high splenic and hepatic uptake (e.g., 18% ID/g of anti-Thy-1.1 in spleen). The lung-to-blood ratio was 5, 10, and 15 for anti-Thy-1.1, anti-ACE, and anti-ICAM-1, respectively. PECAM antibodies displayed low pulmonary uptake in perfusion (2% ID) and in vivo (3–4% ID/g). However, conjugation with streptavidin (SA) markedly augmented pulmonary uptake of anti-PECAM in perfusion (10–54% ID, depending on an antibody clone) and in vivo (up to 15% ID/g). Therefore, ACE-, Thy-1.1-, ICAM-1-, and SA-conjugated PECAM MAbs are candidate carriers for pulmonary targeting. ACE MAb offers a high selectivity of pulmonary targeting in vivo, likely because of a high content of ACE-positive capillaries in the lungs.

drug/gene delivery; antibody-mediated lung targeting; angiotensin-converting enzyme; endothelial antigens

SITE-SELECTIVE DELIVERY of drugs or genes to endothelial cells may provide more effective and specific means for prophylaxis or/and therapies of pathological conditions associated with endothelial dysfunction, such as hypertension, thrombosis, atherosclerosis, acute respiratory distress syndrome, cancer, inflammation, and diabetes (3, 17, 23). Endothelial cells display antigenic and functional heterogeneity in different tissues and organs, either normal or pathologically altered (22, 47, 53, 63). Even in the same organ, endothelial cells may normally exhibit a significant heterogeneity in different segments, e.g., venous vs. arterial (21, 33). It is tempting to postulate that endothelial heterogeneity may be used for more precise targeting of drugs to a defined organ or vascular area. These considerations provide a rationale and framework for the vascular immunotargeting strategies using antibodies directed against endothelial surface determinants as carriers for site-selective drug delivery.

Importantly, pulmonary endothelium represents a privileged vascular target because lungs receive the first passage of the whole cardiac output of venous blood and possess a high density of capillaries (~30% of the total endothelium in the body belongs to the pulmonary vasculature; see Ref. 19). Therefore, even antibodies that do not discriminate between pulmonary and systemic endothelium may accumulate in the lung after intravascular injection. In support of this notion, native and modified antibodies to angiotensin-converting enzyme (ACE; see Refs. 11 and 43), thrombomodulin (TM; see Ref. 31), intercellular adhesion molecule-(ICAM)-1 (48), platelet endothelial cell adhesion molecule (PECAM)-1 (35, 42), and a nonidentified caveola-associated antigen (57) recognize endothelium in vivo, accumulate in the lungs, and may serve as carriers for drug/gene targeting to the endothelium.

None of these antibodies, however, qualify as universal or ideal carriers, since every pathological setting presents unique requirements for a drug delivery system. The need for different carriers to design delivery

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systems adequately serving various therapeutic aims justifies studies of endothelial recognition by antibodies and the quest for new potentially useful carriers. However, a systematic characterization of some potentially useful carriers in terms of their binding to endothelium in the lungs and in extrapulmonary blood vessels is missing in the literature. The goal of the present work was to characterize distribution of the target endothelial antigens in the vasculature of laboratory animals and correlate this information with the data on biodistribution of radiolabeled monoclonal antibodies (MAbs) after systemic injection in vivo.

Theoretically, success of vascular immunotargeting strategies depends on tissue distribution of surface endothelial antigens, their accessibility to carrier MAbs, and affinity of the MAbs. Therefore, evaluation of these parameters is an important component of immunotargeting strategies. To characterize distribution of the antigens in organs, including lungs, we stained rat tissue sections with MAbs and graded semiquantitatively intensity of immunostaining in various vascular areas. To estimate accessibility of the endothelial antigens to circulating MAbs, we determined uptake of radiolabeled MAbs in isolated perfused rat lungs. To evaluate tissue selectivity of the targeting and evaluate potential applicability of the MAbs as drug carriers, we characterized tissue distribution of radiolabeled MAbs in rats after intravascular injection in vivo.

We studied MAbs directed against the following surface endothelial antigens: CD31 (anti-PECAM-1 MAb 164.1), CD54 (ICAM-1 MAb 1A29), CD90.1 (anti-Thy-1.1 MAb OX-7), CD143 (anti-ACE MAb 9B9), and the murine MAb against rat endothelium (MRC OX-43). We also determined the affinity of several PECAM antibodies and their uptake in the lungs. Our results indicate that antibodies directed against ACE, ICAM-1, Thy-1, and PECAM modified with streptavidin (SA) might serve as carriers for vascular immunotargeting to different vascular areas, including lung, heart, and spleen. Of specific interest in the context of lung biology and pathology, anti-ACE MAb offers more selective pulmonary targeting in vivo than other candidate carriers tested in this work, most likely because of a very high content of ACE in the pulmonary capillary endothelium.

MATERIALS AND METHODS

Materials. IODO-GEN was obtained from Pierce (Rockford, IL), and $Na^{125}I$ was from Amersham (Arlington Heights, IL). Dimethyl formamide, TCA, and normal mouse IgG were purchased from Sigma (St. Louis, MO). SA and 6-biotinylaminocaproic acid *N*-hydroxysuccinimide ester (long-arm biotin ester) were purchased from Calbiochem (San Diego, CA).

Antibodies. Properties of the anti-ICAM-1 (CD54) MAb (clone 1A29) were described previously (48, 59). This MAb was purchased from PharMingen (San Diego, CA). Mouse anti-rat endothelium MAb, clone MRC OX-43 (54), was purchased from Serotec (Oxford, UK). Mouse MAb against rat CD90.1 (Thy-1.1; clone OX-7) was from PharMingen. Properties of the anti-ACE MAbs (clones 9B9 and 5F1) have been described previously (9–11). Anti-ACE 9B9 cross-reacts with human, rat, and cat ACE, whereas MAb 5F1 does not cross-react with rat ACE and therefore served as a negative control in immunohistochemistry studies. The following antibodies directed against PECAM-1 (CD31) were used in the study: MAb clone 164.1 (along with a purified chimera protein CD31) was kindly provided by Dr. P. Newman (Blood Research Institute, Milwaukee, WI); MAb clones 4G6 and 37 and polyclonal rabbit antibody "Houston" were kindly provided by Dr. S. M. Albelda (University of Pennsylvania); MAb clone 6E4 was kindly provided by Drs. T. Vlasik and A. Mazurov (Russian Cardiology Research Center, Moscow); MAb clone 62 was provided by Dr. M. Nakada (Centocor; see Ref. 44). Mouse MAb 164.1 was produced using rat PECAM-1 isoform as an immunogen. All other monoclonal and polyclonal antibodies have been produced using human PECAM-1 as an immunogen; however, some clones cross-reacted with rat PECAM isoform.

Immunostaining of rat tissues with anti-endothelial antibodies. Immunoenzymatic detection of ACE and other endothelial antigens was mainly performed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (5), adapted for ACE staining as described (37). In brief, frozen tissue sections were air-dried and fixed in acetone for 10 min at room temperature. Sections were incubated with the primary MAb and then with rabbit anti-mouse Ig (1:40; DAKO) supplemented with a 1:750 dilution of reconstituted lyophilized rat serum (Dianova). This was followed by incubation with the APAAP complex (1:50; DAKO). Each step lasted 30 min at room temperature. Samples were thoroughly washed in Tris- and HCl-buffered saline (pH 7.6) between steps. Alkaline phosphatase substrate reaction with new fuchsin (100 μg/ml) and levamisole (400 μg/ml) was performed for 20 min at room temperature. Sections were counterstained with hematoxylin and mounted in gelatin. Before the systematic study of endothelial antigen distribution, different dilutions of all MAbs were tested on different native tissues to determine the optimal MAb concentration, which was between 1 and 10 μg/ml. Further increase of these concentrations did not increase the maximal intensity of endothelial immunostaining, suggesting saturation of all antigenic sites.

To estimate the level of endothelial antigen expression within the different vessels, a semiquantitative analysis of immunostaining was performed as previously described (25, 52). Recently, this approach was successfully employed for the quantification of ACE expression in kidneys in humans (37). The predominant expression pattern of endothelial antigens was estimated for each vascular segment using the following increments of staining intensity: negative (−), weak (+), moderate (++), or strong (+++). PECAM-1 (CD31) is expressed at high levels in endothelial cells in different vessels (a "pan-endothelial antigen"; see Ref. 49). Accordingly, +++ characterized an intensity of vascular staining with antibodies as strong as the immunostaining with the anti-PECAM (37). Previously, experiments with various concentrations of various anti-ACE MAbs showed that an increase in one immunohistochemical scoring point was equivalent to a 10-fold increase in immunoreactive sites (Franke, unpublished observations). Tissue sections were inspected by two morphologists (Franke and Pauls), and the immunoreactivity score and mean expression were assigned independently without substantial differences. More than 300 cryostat sections of the vascular tree in the main organs of six rats were analyzed in the study.

Radiolabeling of antibodies. In a pilot study, we determined the optimal conditions for radiolabeling of antibodies by iodinating MAb 9B9 using IODO-GEN-coated tubes

(Pierce), IODO-beads (Pierce), or Bolton-Hunter reagent (Amersham). The results of this comparison, shown in Table 1, indicate that the IODO-GEN method provides the best results in terms of effectiveness of radiolabeling and binding capacity of the antibody. Based on this result, we used the IODO-GEN method for radiolabeling of all antibodies used in the study. Briefly, 100 μg of protein were incubated with 100 μCi of $Na^{125}I$ for 30 min on ice. The excess iodine was removed by gel filtration on a PD-10 (Sephadex G-25) minicolumn (Pharmacia, Uppsala, Sweden). Analysis with TCA precipitation of radiolabeled proteins showed that free iodine content in the labeled antibodies did not exceed 5%.

Analysis of binding of radiolabeled PECAM antibodies to immobilized CD31. Plastic wells of "Microtest" plates were coated with human CD31 by overnight incubation at 4°C with 100 ng in BBS (pH 8.1). After elimination of nonbound CD31, wells were incubated with BSA to block nonspecific binding sites. Wells coated with BSA without CD31 served as a control for the specificity of binding. ^{125}I-labeled antibodies were incubated in the wells for 1 h at room temperature. After elimination of nonbound materials, radioactivity in the wells was determined in a gamma counter.

Perfusion of isolated rat lungs. Male Sprague-Dawley rats weighing 170–200 g were anesthetized with pentobarbital sodium (50 mg/kg ip). Lungs were isolated and perfused with a recirculating buffer as previously described (18). Briefly, the trachea was cannulated, and the lungs were ventilated with a humidified gas mixture (Airco, Philadelphia, PA) containing 5% CO_2 and 95% air. Ventilation was done with an SAR-830 rodent ventilator (CWE) at 60 cycles/min, a tidal volume of 2 ml, and an end-expiratory pressure of 2 cmH_2O. The thorax was then opened, and a cannula was placed in the main pulmonary artery through the transected heart. The lungs were isolated from the thorax and transferred to a water-jacketed perfusion chamber maintained at 37°C. There was no interruption of ventilation during this process, and interruption of the lung perfusion was <5 s. Perfusion through the pulmonary artery was maintained by a peristaltic pump at a constant flow rate of 10 ml/min. The perfusate (45 ml) was Krebs-Ringer buffer (KRB, pH 7.4) containing 10 mM glucose and 3% fatty acid-free KRB-BSA solution. The perfusate was filtered through a 0.4-μm filter before perfusion to eliminate particles. One microgram of radiolabeled MAb was added to the perfusate. After 1 h of perfusion with the radiolabeled protein, the lungs were perfused with a single-pass perfusion for 5 min with perfusate that did not contain radiolabeled protein to eliminate nonbound radioactivity. At the end of experiment, the lungs were removed from the chamber, rinsed with saline, and blotted with filter paper; the lung wet weight was determined, and its radioactivity was measured in a gamma counter and expressed as a percentage of perfused radioactivity per lung. The radioactivity associated with parts of the perfusion system was determined in a prototype experiment. This parameter did not exceed 0.01% of the total radioactivity added to the perfusate. Accordingly, this value has been ignored in the analysis of the results.

In vivo administration of radiolabeled antibodies and biodistribution studies. Male Sprague-Dawley rats were injected with ^{125}I-labeled MAb via the tail vein under halothane anesthesia. A design, protocol, and analysis of a typical experiment have been described previously (11). Briefly, animals were killed 2 h postinjection, and the internal organs were dissected, washed with saline, blotted dry, and weighed. Tissue radioactivity in organs was determined in a gamma counter; 1 ml of blood, a piece of liver (~1 g), and the entire nonhomogenized lung, spleen, kidney, and heart were used for measurement.

The results of ^{125}I measurements in the organs were used to calculate three parameters characterizing related yet different aspects of ^{125}I-MAb biodistribution and targeting. First, the percentage of the injected dose in an organ (%ID) characterizes a total value of antibody uptake in an organ. This parameter shows biodistribution and effectiveness of antibody targeting. Second, the percentage of the injected dose per gram of tissue (%ID/g) permits comparisons of uptake of the antibodies in different organs. Without such normalization, uptake in larger organs (e.g., liver) may look extremely large, even when compared with a very specific and effective uptake in a relatively small organ (e.g., lung or heart). Therefore, this parameter evaluates tissue selectivity of an antibody uptake. Importantly, this parameter also permits comparison of the data obtained in different animal species. These two parameters (%ID and %ID/g) provide different absolute values in many organs (including lung, which is not 1 g exactly in rats), as reflected in the difference between values in Table 4 and Fig. 3. Third, the ratio between percent ID per gram in an organ and that in blood gives the localization ratio (LR). This parameter compensates for a difference in the blood level of circulating radiolabeled antibody (e.g., due to the different rate of uptake by clearing or target organs). Therefore, LR allows a more objective comparison of targeting between different carriers, which have different rates of blood clearance.

Table 1. *Comparison of different methods for radioiodination of anti-ACE MAb 9B9*

Method of Iodination	Bolton-Hunter	IODO-GEN	IODO-Beads
Labeling yield, %	71	41	29
Pulmonary uptake, %ID	23.3 ± 1.8	27.5 ± 3.2	7.25 ± 0.04

Values are means ± SE; n = 3 experiments. The effectiveness (yield) of the radioiodination was determined as percent of protein-coupled ^{125}I recovered after the labeling. Biological activity of radioiodinated antibody was determined in vivo by injecting 1 μg of ^{125}I-labeled monoclonal antibody (MAb) 9B9 in rats. Pulmonary uptake, reflecting binding of the antibody to angiotensin-converting enzyme (ACE) in the lungs, was determined 1 h after intravenous injection and was expressed as percent of injected dose in the lung (%ID). Note that IODO-GEN method permits labeling with minimal extent of MAb 9B9 inactivation (the highest value of pulmonary accumulation among other methods) with an intermediate effectiveness of the labeling.

RESULTS

Immunohistochemical characterization of antigen distribution in rat tissues. In the first part of this work, we characterized immunostaining of ACE (CD143), Thy-1.1 (CD90.1), ICAM-1 (CD54), PECAM-1 (CD31), and rat OX-43 antigen in rat organs using MAbs employed for in vivo studies (see below). The aims of this study were to *1*) compare binding of different MAbs in the organs under identical conditions and evaluate the total content of MAb binding sites in the organs; *2*) visualize cell types recognized by MAbs; and *3*) reveal potential heterogeneity of the MAbs binding to endothelial cells in different types of blood vessels in the vasculature. For maximal epitope preservation, we used fresh frozen tissues for immunostaining sections. A preliminary study showed that acetone fixation employed in the study, a standard procedure for tissue

preparation for immunostaining using fresh frozen tissues, does not alter the epitopes.

All tested MAbs except anti-PECAM MAbs 6E4 and 4G6 labeled endothelial cells in the rat tissues (Table 2). This result indicates that they cross-react with the rat isoform of human counterpart antigens and therefore are suitable for the following experiments in perfusion of isolated rat lungs and in vivo. When incubated with the tissue sections, MAbs also displayed binding to other cell types than the endothelium in certain organs. For example, Thy-1.1 was found in lymphocytes in the spleen (Fig. 1*E*), PECAM-1 was found in macrophages and platelets in the spleen (Fig. 1*F*), ICAM-1 was found in macrophages (data not shown), and ACE was found in the epithelial cells in the kidneys and epididymis (data not shown).

Studied MAbs displayed different staining patterns in the endothelium in systemic and peripheral vessels of a different caliber (reactivity in the pulmonary vasculature is described below). For example, anti-Thy-1.1 MAb displayed positive staining in the capillary but not in the arterial and venous endothelium (Figs. 1*A* and 2*D*). In contrast, anti-ACE MAb displayed positive staining in arterial endothelial cells (Fig. 1*B*), whereas capillary endothelium staining was sparse or even negative in most organs, except the lung. Antibodies against ICAM-1 and PECAM-1 displayed a more homogeneous immunostaining pattern in endothelial cells in the vessels of different caliber throughout the vascular system (Fig. 1*C*). This pattern of anti-ICAM and anti-PECAM MAbs correlates well with a relatively homogeneous distribution of their antigens in vasculature in diverse animal species described in the literature (Refs. 14 and 49, respectively). Table 2 summarizes the data on the distribution of the studied antigens in endothelial cells in different types of vessels.

Because capillaries represent the most extended vascular surface area in many target organs, we specifically characterized the MAb immunostaining pattern in the capillaries in different organs. In this type of blood vessels, ACE antigen displayed the most striking pulmonary selectivity: 100% of alveolar capillaries were strongly labeled by the anti-ACE MAb 9B9 (Fig. 2*A*). In sharp contrast, hepatic and renal capillaries were negative, and only 5–20% of the capillaries in other organs were positive for anti-ACE MAb immunostaining. Other tested antibodies also showed labeling of pulmonary capillary endothelial cells (Fig. 2, *B–E*). However, their antigens were well presented in the capillaries of other organs as well, except for a relatively weak immunostaining of ICAM-1 in cardiac and PECAM-1 in splenic capillaries (Table 3).

Uptake of ^{125}I antibodies in the isolated perfused rat lung. Exposure of tissue sections to antibodies for a sufficient time allows binding to a maximal number of tissue epitopes, both intra- and extracellular. Therefore, immunochemical study does not provide an insight into the antigen's ability to harbor ligands circulating in the bloodstream. To address this important issue directly and estimate accessibility of the endothelial antigens to circulating antibodies in the absence of potentially confusing systemic and blood effects, we measured the uptake of ^{125}I-MAbs in isolated rat lungs perfused with blood-free buffer. Figure 3 shows that anti-ICAM-1 MAb 1A29, anti-ACE MAb 9B9, and anti-endothelial MAb MRC OX-43 accumulated in rat lungs at a similar extent, ranging from 20 to 25% ID. Noteworthy, pulmonary uptake of anti-Thy-1 MAb OX-7 was extremely high (45% ID). Control ^{125}I-labeled IgG did not accumulate in the perfused rat lungs (0.4 ± 0.04% ID), thus confirming the specificity of accumulation of the radiolabeled anti-endothelial ^{125}I-MAbs. Anti-PECAM MAb 164.1 demonstrated low pulmonary uptake (1.9 ± 0.8% ID). The issue of PECAM antibody affinity and targeting is addressed separately in *Binding of PECAM MAbs to purified CD31 antigen and to pulmonary endothelium.*

Distribution of ^{125}I-MAb in the tissues after intravenous injection in rats in vivo. Table 4 shows the data for tissue distribution of ^{125}I-MAbs 2 h after intravenous injection in intact anesthetized rats. The blood level of control IgG was close to 3% ID/g. Taking into account that, in an average-sized rat (250 g), total blood volume is ~15–18 ml, this result indicates that ~50% of injected IgG circulates in the bloodstream. The level of anti-endothelial ^{125}I-MAbs in the blood was significantly lower than that of the control IgG. This result likely reflects their binding to the endothelium (and, perhaps, other target cells in the tissues accessible to the bloodstream), leading to depletion of the circulating pool.

Control ^{125}I-IgG did not accumulate in any organ. Absolute values of IgG uptake in the tissues were significantly lower than its blood level. Therefore, the IgG tissue-to-blood ratio (LR) was <0.5 in all tissues,

Table 2. *Endothelial reactivity pattern of different MAbs within the systemic vasculature of the rat except the vessels of lung*

Antigen	Antibody	Aorta	Arteries	Arterioles	Capillaries	Veins
Unknown	MRC OX-43	+++	+++/+	+++/+	+++/−	+++
PECAM-1 (CD31)	164.1	+++	+++	+++	++/−	+/++
Thy-1.1 (CD90.1)	OX-7	−	−	−/++	+++/−	+/−
ICAM-1 (CD54)	1A29	++	++/−	+/−	++/−	++/−
ACE (CD143)	9B9	+++	+++	+++	−/+	++/−

PECAM, platelet endothelial cell adhesion molecule; ICAM, intercellular adhesion molecule. The degree of immunoreactivity is scored from − (no reactivity) to +++ (strong reactivity), whereby dash (/) indicates variations in immunoreactivity within the same types of vessels. Note the relative homogeneous endothelial distribution of CD31, e.g., compared with CD90.1.

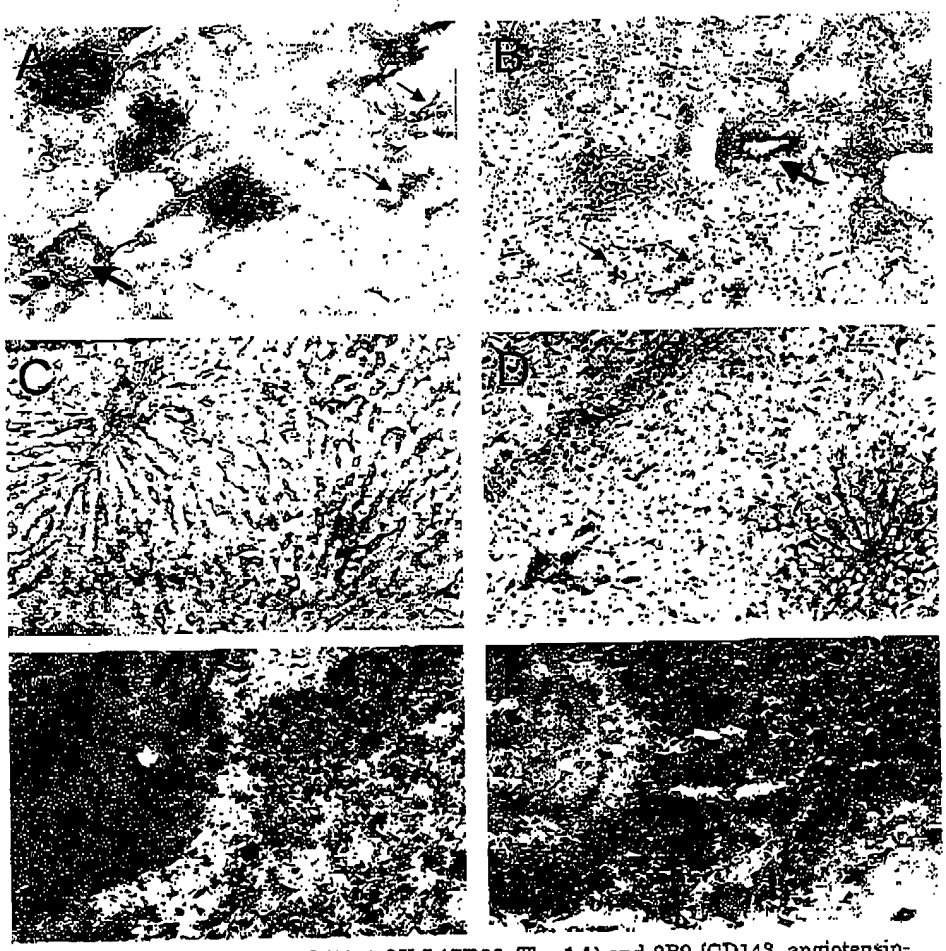

Fig. 1. Immunoreactivity of monoclonal antibodies (MAbs) OX-7 (CD90, Thy-1.1) and 9B9 [CD143, angiotensin-converting enzyme (ACE)] on renal tissue (*A* and *B*), of 1A29 [CD54, intercellular adhesion molecule (ICAM)-1] and OX-43 (unknown antigen) on liver tissue (*C* and *D*), and of OX-7 (CD90.1, Thy-1.1) and 1641 [CD31, platelet endothelial cell adhesion molecule (PECAM)-1] in splenic tissue of rat (*E* and *F*). APAAP, alkaline phosphatase anti-alkaline phosphatase. Magnification, ×150. The MAb to Thy-1.1 selectively decorated endothelial cells of the glomerule and some perivascular cells and capillaries of the medulla renalis (small arrows). In contrast, endothelial cells of arteries (large arrow), veins, and cortical capillaries were negative or faintly labeled. The MAb to ACE showed strong reactivity with endothelial cells of large and small arteries (large arrow), but most endothelial cells of the glomerule (G), veins, and the capillary network were negative. Note the faint ACE expression in some epithelial brush borders of the distal proximal tubule (small arrows), a finding that is unique for the rat kidney because tubular epithelium express high amounts of ACE in other species. The anti-ICAM-1 MAb was homogeneously reactive with all sinusoidal endothelial cells of the liver parenchyma. Heterogeneous expression of the OX-43 antigen within the same capillary network of liver is seen. Note the typical starlike decoration of sinusoidal lining cells around the central veins. Within the peripheral and midzone of the lobule, only some von Kupffer cells were marked. In spleen, strong nonendothelial labeling is seen for the MAb to Thy-1.1, which also reacts with lymphocytes. In addition to endothelial cells, PECAM-1 is expressed by macrophages and thrombocytic particles, causing strong immunoreactivity of the red pulp.

including the lungs (LR <0.3). None of the tested antibodies accumulated in the intestine. Renal uptake was low for all antibodies, although anti-PECAM-1 and anti-Thy-1.1 MAbs displayed a slightly higher renal uptake than control IgG. Anti-endothelial MAb MRC OX-43 demonstrated a marked uptake in the spleen and significant uptake in the heart, whereas its pulmonary accumulation was negligible.

The data in Table 4 are expressed as percentage of the injected dose per gram to allow comparison between different organs. To evaluate total uptake in tissues, one has to multiply the data on organ weight (e.g., on average, 15 and 12 g for blood and liver). Therefore, it is relatively easily to take into account the blood pool, ranging from 50 to 20% ID (IgG and anti-ACE, respectively), and hepatic uptake, ranging from 40 to 15% ID (anti-ICAM and anti-ACE, respectively). However, as seen with tracing of radiolabeled proteins in vivo, the balance of injected tracers in major organs and blood does not usually sum up to 100% ID and averages 60–80% ID for many MAbs. The main reason is that it is difficult to accurately determine uptake in skin, muscles, and other nonmeasured parts of the body.

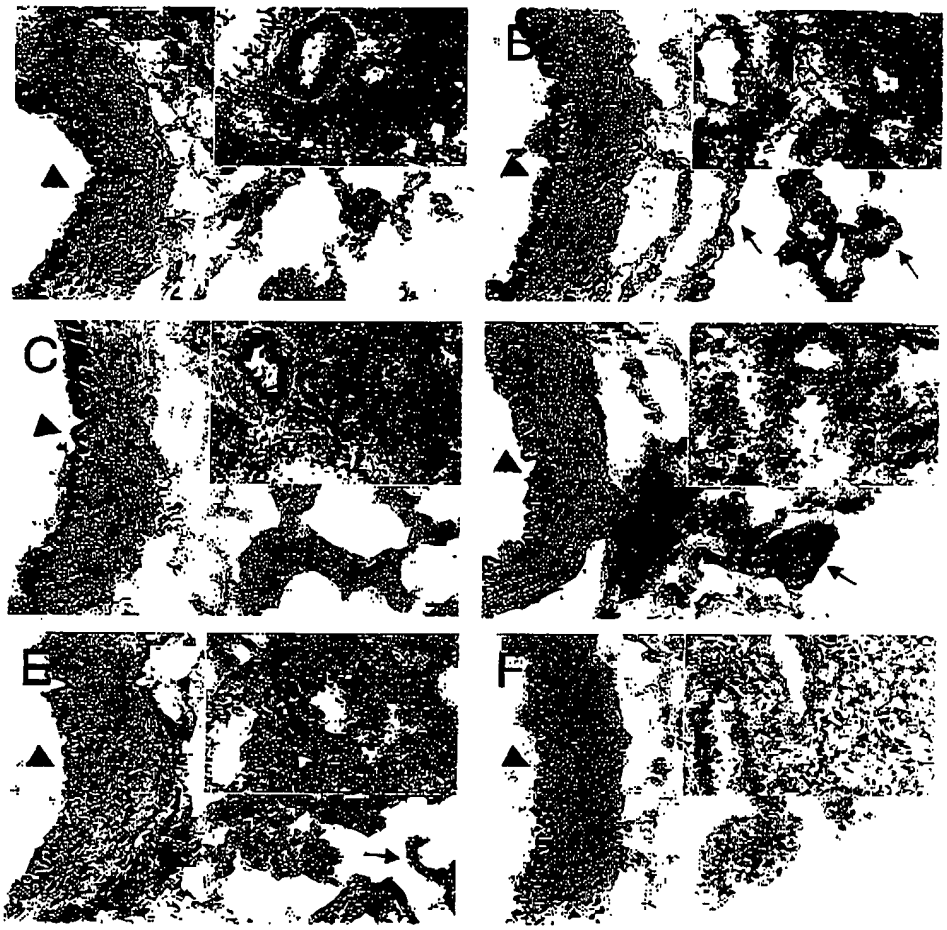

Fig. 2. Immunoreactivity of MAbs 9B9 (CD143, ACE), 1A29 (CD54, ICAM-1), 164.1 (CD31, PECAM-1), OX-7 (CD90, Thy-1.1), OX-43 (unknown antigen), and 5F1 (control IgG) on serial sections of lung tissue of the rat (*A–F*). The same pulmonary artery is shown on *left* while *insets* show general reactivity pattern of APAAP. Magnification, ×300, except in *insets*, where magnification is ×150. Endothelial cells of lung tissue were homogeneously and strongly labeled by the MAb to ACE, encompassing those of pulmonary arteries (arrowhead) and alveolar capillaries. The MAbs to ICAM-1 show binding to endothelial (arrowhead) and epithelial (arrows) cells in the alveolar compartment. PECAM-1 was expressed by endothelial cells of the pulmonary artery (arrowhead) and alveolar capillaries (*inset*), although intensity of expression appears to be weaker in the latter. The heterogeneous endothelial expression of Thy-1.1 is obvious in lung tissue. Although endothelial cells of pulmonary arteries lacked immunoreactivity (arrowhead), alveolar capillaries were partially labeled. Note the additional perivascular reactivity (arrow), indicating expression of Thy-1.1 by some pericytic cells. The MAb to the OX-43 antigen weakly decorated endothelial cells of the lung (arrowhead). Endothelial labeling was somehow blurred by additional stromal reactivity (arrow). The MAb 5F1 to human ACE did not cross-react with ACE of the rat; therefore, this MAb served as a negative control.

Many MAbs employed in the study displayed the significant accumulation in the lungs. Notably, among other tested antibodies, anti-ACE MAb 9B9 displayed the highest absolute value (16% ID/g) and selectivity of the pulmonary uptake, with no detectable uptake in other organs. Anti-ICAM-1 and anti-Thy-1.1 MAbs displayed moderate and modest pulmonary uptake (4 and 6% ID/g) and also accumulated in liver and spleen. Anti-Thy-1.1 MAb OX-7 displayed a remarkably high uptake in the spleen (18% ID/g). Uptake in the nonpulmonary targets (correlating with a high content of ICAM-1 and Thy-1.1 in the nonpulmonary capillaries; Table 3) led to a pronounced depletion of the blood pool of ^{125}I-anti-Thy-1.1 and ^{125}I-anti-ICAM-1. As a result, pulmonary LR of these ^{125}I-MAbs was close to 5 and 15, respectively. Taken together, the results presented indicate that antibodies against ICAM-1 and Thy-1.1 accumulate promiscuously in many organs, including the lungs, whereas anti-ACE accumulates selectively in the lungs.

Table 3. *Distribution of antigen-positive capillaries in pulmonary vs. systemic circulation*

Antigen	Antibody	Lung	Liver*	Kidney	Spleen	Heart	Intestine
Unknown	MRC OX-43	100	20	100	ND	100	60
		+	++	+++		+++	+
PECAM-1 (CD31)	164.1	100	100	100	25	100	100
		+	+	++	+	+	+
Thy-1.1 (CD90.1)	OX-7	80	0	50	30	50	80
		+	−	++	+	++	+++
ICAM-I (CD54)	1A29	100†	100	100	50	10	20
		+	++	+	++	++	+
ACE (CD143)	9B9	100	0	0	20	10	5
		+++	−	−	+	+	+

The percentage of immunoreactive endothelial cells in the capillary system of different organs is given in numerical values; the intensity of antigen expression is indicated by score values from − (no expression) to +++ (strong expression). *Sinusoids of liver parenchyma, which represent >99% of the endothelial surface of liver. ND, not determined (because of strong background immunoreactivity concerning interstitial and inflammatory cells). †Difficult to assess in alveoli of rat lung because CD54 was also found in regular alveocytes.

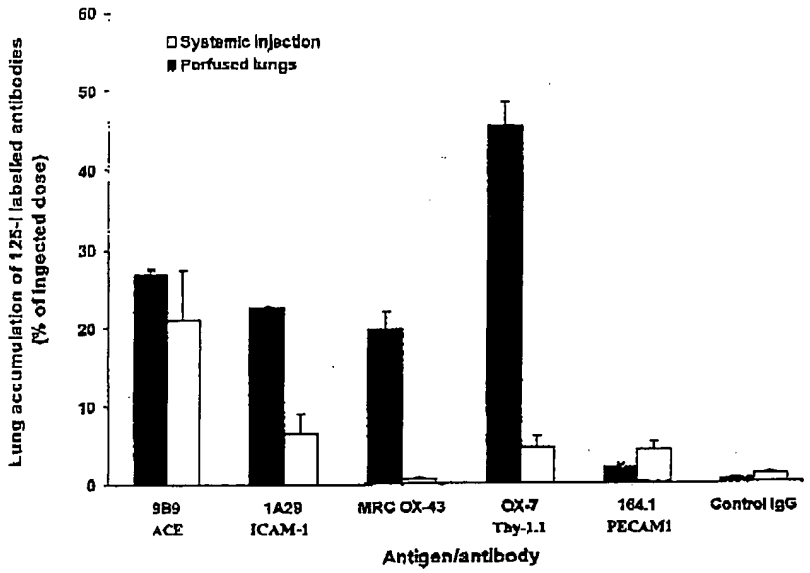

Fig. 3. Lung accumulation of ^{125}I-labeled MAbs to endothelial antigens in isolated perfused lung and after systemic injection. ^{125}I-labeled MAbs (300,000 counts/min, 1 μg) were added to perfusate [isolated perfused lungs (IPL)] or injected into the tail vein. Lung uptake was estimated 2 h after perfusion or after injection and is expressed as percent injected dose (means + SD; n = 3 experiments).

Binding of PECAM MAbs to purified CD31 antigen and to pulmonary endothelium. Figure 3 shows that anti-PECAM MAb 164.1 displayed a relatively modest, nonselective uptake in the lungs and other organs in vivo (3.5% ID/g in the lung, LR >10). Therefore, MAb 164.1 displayed slightly higher pulmonary uptake in vivo than in the perfused rat lungs (2% ID). The blood plasma volume in rats (6 ml) is seven times smaller than that of the perfusion buffer (45 ml). Therefore, an apparent contradiction between MAb 164.1 pulmonary uptakes in vivo and in perfusion can be reconciled based on the assumption that the affinity of the MAb 164.1 clone is a limiting factor. In general, the affinity of MAbs binding to endothelial antigens is a critically important parameter of the targeting. Capitalizing on the availability of purified human CD31 (kindly provided by Dr. P. Newman), we performed a more systematic study of the binding profile of different anti-PECAM clones.

Figure 4 shows typical binding curves of radiolabeled MAb (MAb 6E4; *A*, and MAb 62; *B*) and polyclonal antibodies (rabbit Ab Houston) to immobilized CD31. The bottom curves in Fig. 4; *C*, represent binding to control wells coated with albumin. Binding of ^{125}I-IgG to CD31-coated wells did not differ from binding of ^{125}I-anti-PECAM to albumin. These controls help to appreciate a high specificity of anti-PECAM binding to CD31. To characterize the affinity of PECAM antibodies, the Scatchard analysis of the binding curves (linear regression between bound ligand and the bound-to-free ratio) has been performed. Figure 4, *insets*, shows results of this regression and indicate that binding curves of all anti-PECAM clones display a fairly good linearization pattern, permitting determination of their affinity constants (K_a).

Table 5 shows that K_d values determined for five different clones of anti-PECAM varied from relatively high (5 and 10 nM for MAbs 37 and 62) to relatively low (250 nM for polyclonal antibody Houston). However, despite significant variations in affinity, all tested PECAM antibodies displayed relatively low uptake in the perfused rat lungs (2.0–2.5% vs. 0.6 ± 0.1% ID/g for control IgG) and in rat lungs in vivo (2.5–3.6% ID/g). In the previous study, we observed that coupling to SA augments binding and internalization of biotinylated PECAM antibodies by endothelium (42). In good agreement with this early observation, experiments in perfused rat lungs showed that conjugation with SA augmented pulmonary uptake of biotinylated Ab Houston and MAb 62 to 40–50% ID/g. SA had no effect on pulmonary uptake of biotinylated control IgG. Conjugation of biotinylated MAb 164.1 with SA markedly augmented its pulmonary uptake in perfused rat lungs (10% ID/g) yet had no effect on pulmonary uptake in

Table 4. *Biodistribution of ^{125}I-MAbs in rat after systemic injection*

Antigen	Antibody	Blood	Lung	Liver	Kidney	Spleen	Heart	Intestine
Unknown	MRC OX-43	1.12 ± 0.34	0.47 ± 0.12	0.69 ± 0.22	0.93 ± 0.35	6.67 ± 2.09*	1.89 ± 0.42*	0.62 ± 0.10
PECAM-1 (CD31)	164.1	0.27 ± 0.05	3.55 ± 0.78*	2.93 ± 0.49*	1.47 ± 0.38*	1.74 ± 0.26*	1.30 ± 0.59*	0.21 ± 0.03
Thy-1.1 (CD90.1)	OX-7	0.77 ± 0.25	3.73 ± 1.10*	1.73 ± 0.33*	1.57 ± 0.27*	18.13 ± 4.01*	0.14 ± 0.03	0.09 ± 0.04
ICAM-1 (CD54)	1A29	0.34 ± 0.02	5.52 ± 1.99*	2.79 ± 0.44*	0.80 ± 0.06	3.22 ± 0.60*	0.49 ± 0.14	0.32 ± 0.04
ACE (CD143)	9B9	1.67 ± 0.62	15.65 ± 5.38*	0.65 ± 0.18	0.47 ± 014	0.48 ± 0.16	0.45 ± 0.13	0.44 ± 0.14
Control	5F1	3.79 ± 0.46	0.87 ± 0.21	0.94 ± 0.20	1.04 ± 0.07	0.46 ± 0.11	0.78 ± 0.09	0.51 ± 0.05

Data are means ± SD; n = 3 experiments. Control, ^{125}I-mouse IgG. Units are %ID/g tissue. Rats were injected via tail vein with ^{125}I-MAbs (300,000 counts/min, 1 μg). After injection (2 h), animals were killed, internal organs were dissected, washed with saline, and weighed; and radioactivity in organs was measured in a Rack-Gamma counter. *Values of accumulation statistically significant from control IgG.

vivo. However, MAb 62 conjugated with SA displayed a very high pulmonary uptake after injection in intact rats. The absolute value of SA-MAb 62 uptake attained 15% ID/g, close to that of anti-ACE MAb 9B9 (see Fig. 3). Therefore, the anti-PECAM-SA conjugate represents a good candidate for vascular immunotargeting to pulmonary endothelium.

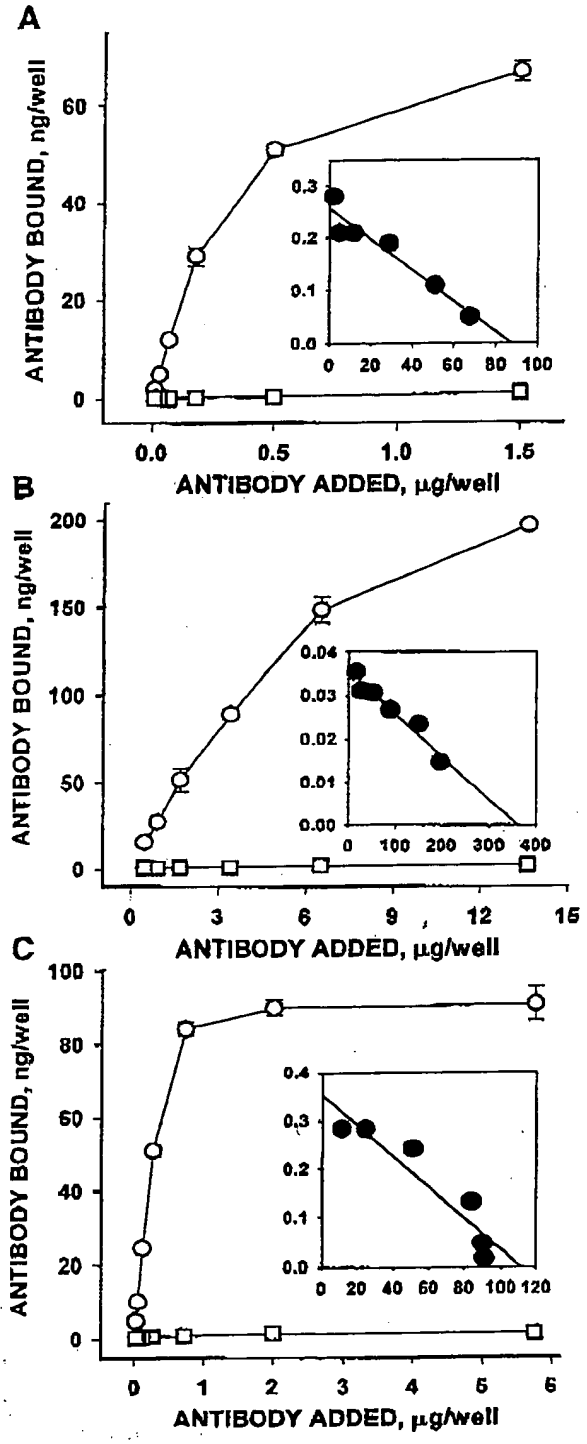

Fig. 4. Binding of different anti-PECAM MAbs to immobilized human CD31. Radiolabeled anti-PECAM antibodies were incubated in the wells coated with CD31 (○) or albumin (□) for 1 h at room temperature. The binding was determined after extensive washing of nonbound antibodies and is expressed as means ± SD ($n = 3$). *Insets* show linearization of the CD31 binding curves in Scatchard graphs (y-axes show the bound-to-free ratio, x-axes show bound MAb).

DISCUSSION

Pulmonary endothelium represents an important and privileged target for site-specific delivery of therapeutic agents or genes. Immunotargeting strategies are based on conjugation (chemically or via gene engineering) of a drug (enzyme, gene) with carrier antibodies that recognize specific antigens on the surface of the target cell (51). Antibodies directed against surface endothelial antigens represent potential carriers for the targeting of drugs or genes to the pulmonary endothelium. For example, TM antibody recognizes endothelial cells in vitro and in vivo, accumulates in the murine lung after intravascular injection (31), and is capable of delivery of liposomes or genetic material to the pulmonary endothelium (36, 60). The therapeutic applicability of anti-TM is, however, restricted by the intracellular degradation of internalized TM ligands (26, 41) and by suppression of the anticoagulant function of TM by anti-TM (16). Schnitzer (57) reported the pulmonary accumulation of an antibody recognizing the nonidentified caveola-associated antigen in rats in vivo. However, both the function of this antigen and potential effects of the antibody remain to be characterized more rigorously to evaluate their therapeutic potential. Antibodies recognizing surface adhesion molecules such as PECAM-1, ICAM-1, E-selectin, P-selectin, and vascular cell adhesion molecule-1 also represent a class of potentially useful carriers (32, 48, 58), although in vivo characterization of these antibodies requires more rigorous study.

To further extend and better characterize the list of potential antibody carriers, in this work we studied binding of antibodies against ACE, PECAM-1, ICAM-1, and Thy-1.1 to their antigens in tissue sections, in the perfused rat lungs, and in vivo. The primary goal of this work was to directly compare different MAb carriers in standard experimental models. To accomplish this goal, we employed certain specific experimental conditions and parameters. First, to facilitate evaluation of total binding of MAbs in the tissues, we did not inflate lungs harvested for immunochemical study. A more compact tissue of hypoventilated lung permitted us to compare the gross binding of different MAbs in comparative study. This approach might preclude a more precise localization of the antigens in specific cell types and compartments (e.g., intracellular vs. surface). Electron microscopy or use of the avidin-biotin couple could, theoretically, provide an insight into surface localization and accessibility of the antigens [e.g., see previous publications on this subject (40, 42)]. However, electron microscopy data are difficult to collect and analyze quantitatively in a significant number of animals. On the other hand, accessibility to biotinylating agents (small molecules, ~400 Da) may be higher than that to large antibody carriers (IgG, 180,000 Da). Therefore, we believe that, in the context

Table 5. *Binding of ^{125}I-PECAM antibodies to purified CD31 and their pulmonary uptake in perfused rat lungs and in vivo*

Antibody	IgG	Antibody Houston	MAb 62	MAb 164.1	MAb 6E4	MAb 37	MAb 4G6
K_d, nM	NA	250*	10*	ND	20	5*	30*
b-MAb uptake in IPL, % ID/g	0.6 ± 0.1	2.5 ± 0.8*	2.3 ± 3.1*	2.1 + 0.3*	ND	ND	ND
SA/b-MAb uptake in IPL, % ID/g	0.5 ± 0.2	53.5 ± 9.3*	39.5 ± 3.1*	10.5 + 1.4*	ND	ND	ND
b-MAb uptake in vivo, % ID/g	2.3 ± 0.7	ND	3.6 ± 1.1*	2.6 ± 0.2*	ND	ND	ND
SA/b-MAb uptake in vivo, % ID/g	2.1 ± 1.1	ND	14.5 ± 2.9*	1.4 ± 0.5*	ND	ND	ND

Values are means ± SE. SA, streptavidin; IPL, isolated perfused lungs. All antibodies have been produced using human PECAM-1 as an immunogen. Affinity of binding to PECAM-1 varies from relatively high (MAb 37 and 62) to low (polyclonal antibody Houston). †Binding affinity of MAb 4G6 and MAb 37 was determined by direct RIA similar to other antibodies, except that human umbilical vein endothelial cells were used as an antigen instead of purified CD31 (binding was performed at 4°C). Antibodies MAb 6E4 and MAb 4G6 did not display cross-reactivity with the rat PECAM isoform. Please note that biotinylated (b) antibodies were used in this study; a minor covalent modification of the antibodies in the course of biotinylation may explain subtle differences between the data on pulmonary uptake shown here and elsewhere in the report (e.g., Table 4 and Fig. 3). NA, not applicable; ND, not determined. *Significantly different from IgG control.

of drug/gene delivery, a direct measurement of the tissue uptake of radiolabeled MAbs provides the most accurate information about accessibility of the target antigens to potential carriers.

Analysis of immunotargeting (i.e., ^{125}I-MAb uptake in different organs) can be performed in several ways, including normalization of MAb uptake per surface vascular area in the organs. However, literature on the surface area of vasculature in different organs is extremely fragmentary, contradictory, and confusing. For example, published data of the vascular surface area in the lungs varied from 38 (8) to 300 (30) m^2 and even 5,000 m^2 (12) in humans and from 2,500 cm^2/g of lung (7) to 4,800 cm^2/g (62) in rats (normalized to a 300-g rat). The data for other organs are even more fragmentary and conflicting (8) and do not allow an accurate and reliable normalization of MAb uptake to the surface area. It is tempting to normalize an antibody uptake to distribution of a pan-endothelial marker, but it raises a question about what such a marker might be. Different antigens, including ACE and PECAM-1, have been proposed as pan-endothelial markers for the sake of interpretation of biodistribution of radiolabeled antibodies in vivo (48, 61). However, an average or "generic" endothelial cell is a constituency that probably does not exist in a real vasculature. For example, it is difficult to exclude that endothelial cells in different vascular areas (even in the same organ) can possess different numbers of copies of PECAM-1. A systematic, morphometric study of surface density of PECAM in the vasculature has yet to be performed. Because the goal of our research is to develop a drug delivery strategy, we used more standard, conventional pharmacokinetic parameters (%ID and %ID/g and LR) that permit comparison between target and nontarget uptake of different agents. From the drug delivery standpoint, percent ID and percent ID per gram characterize the concentration of the delivered drug in target and nontarget organs and therefore permit comparison of local and systemic effects. Speculatively, a relatively high pulmonary uptake of any given antibody could reflect the simple fact that the lung vascular area is larger than that in other organs. However, the vascular surface within the same organ is identical for all studied antibodies. Therefore, analysis of pulmonary uptake of nonspecific control IgG and antibodies directed against pan-endothelial antigens (e.g., ICAM-1) implies that a high pulmonary uptake of anti-ACE likely reflects a specifically high presentation of ACE in the pulmonary capillaries.

ACE is a transmembrane ectopeptidase localized on the luminal surface of the vascular endothelium (2, 55). Anti-ACE MAb 9B9 recognizes rat and human ACE and accumulates in rat lung with high specificity and effectiveness (20–30% ID; see Ref. 11). Recently, we published results of a semiquantitative immunohistochemical study that described a very heterogeneous distribution of ACE in human endothelial cells along the vascular tree in humans. Although the endothelium in small arteries and arterioles displayed a strong ACE expression, the endothelium in aorta, large arteries, and veins displayed poor expression or complete lack of ACE. In the extrapulmonary capillaries, only 10–20% were positive by anti-ACE staining, whereas 100% of pulmonary capillaries displayed strong immunoreactivity with anti-ACE MAb (20, 25, 37).

Results of the present study indicate that, in contrast to the human endothelium, rat endothelium displays more homogeneous and strong ACE expression in large vessels of all types, in either arteries or veins. However, anti-ACE reactivity in rat capillaries and small veins was similar to that seen in human tissues; ACE staining was positive in ~10% of capillary endothelial cells in extrapulmonary tissues, whereas 100% of pulmonary capillaries expressed ACE (Table 3). In good agreement with ACE distribution in the rat vasculature, the radiolabeled MAb 9B9 displayed a tissue-selective accumulation in the lung (Table 4 and Fig. 3).

Noteworthy, our previous data documented that pulmonary uptake of MAb 9B9 does not depend on the route of administration; therefore, it is not a result of the first-passage uptake in the lung (11). In the present study, anti-ACE MAb 9B9 demonstrated similar values of pulmonary uptake in vivo and in the perfused lung. This result excludes the contribution of blood or systemic effects in pulmonary uptake of MAb 9B9 and confirms that it represents a good carrier for the tissue-selective drug/gene delivery to the pulmonary endothe-

lium. The fact that radiolabeled ACE inhibitors (small molecules, 500 Da) accumulate in isolated perfused lung (29) and in whole animals (28) with the same efficacy as the MAb 9B9 (IgG, 170,000 Da) suggests that the accessibility of the ACE epitope for MAb 9B9 is close to ideal. Endothelial cells internalize anti-ACE MAb 9B9 (40). Thus anti-ACE may be useful for intracellular delivery. Anti-ACE MAbs cause no detectable harmful effects in laboratory animals or in normotensive patients. It might be especially attractive for targeting of drugs/genes for treatment of pulmonary hypertension. In this disease, local overexpression of ACE (i.e., target for anti-ACE MAb) in distal, small pulmonary arteries (39) may play a role in the muscularization of these vessels, one of the earliest and most consistent features of pulmonary hypertension (38). At the present time, MAb 9B9 is one of the potentially most useful carriers for pulmonary targeting.

A second potential carrier studied in this work was MAb 1A29, which recognizes the transmembrane endothelial surface adhesion molecule ICAM-1 (48, 59). Endothelial cells constitutively express ICAM-1, yet cytokines and other inflammatory mediators cause further stimulation of its expression (50). In the normal perfused rat lung, MAb 1A29 accumulates to the same extent as MAb 9B9 (Fig. 3). However, the pulmonary accumulation of MAb 1A29 in vivo is markedly less selective than that of MAb 9B9 (Table 4). A more promiscuous pattern of ^{125}I-anti-ICAM biodistribution correlates with a more uniform distribution of ICAM-1 in the vasculature in different tissues (Fig. 1 and Tables 2 and 3). Significant accumulation of MAb 1A29 in the liver correlates well with its high content in the hepatic vasculature. It is likely that depletion of the circulating pool of anti-ICAM-1 by nonpulmonary targets limits its pulmonary uptake. Our preliminary studies imply that injection of saturating doses of radiolabeled anti-ICAM-1 (e.g., 10 μg in mice) permits pulmonary accumulation of 10–15% ID/g (Murciano JC and Muzykantov VR, unpublished observations). Thus anti-ICAM-1 represents an attractive carrier for vascular immunotargeting. For example, our recent study documented that anti-ICAM-catalase conjugate binds to the pulmonary endothelium and protects it against oxidative insult similarly to anti-ACE-catalase (1). In addition, anti-ICAM-1 may be useful for drug delivery to inflammation-engaged endothelium. Results from Granger's laboratory (48) indicate that radiolabeled anti-ICAM accumulates more effectively in the lungs of cytokine-challenged animals.

Another surface adhesion molecule tested in this study was PECAM-1, a member of the Ig superfamily expressed constitutively in endothelial cells at the level of several million copies per cell (46). Qualitatively, PECAM-1 immunostaining in the rat tissues was even more pronounced than that of ACE and ICAM-1 (Figs. 1 and 2). Semiquantitative analysis of immunostaining showed that PECAM-1 is homogeneously distributed throughout all larger vessels, yet PECAM staining is detectably weaker in the capillaries (Tables 2 and 3). This observation supports the notion that the term a "pan-endothelial marker" must be used very carefully.

Recent results from several laboratories show that different PECAM antibodies coupled to enzymes or genetic material accumulate in the lungs in vivo in different animal species and can be used as carriers for the delivery of drugs and genes to the pulmonary endothelium (4, 35, 42, 56). In the present study, radiolabeled anti-PECAM displayed a significant, yet rather modest, pulmonary uptake after intravenous injection in rats (Tables 4 and 5) and a relatively poor uptake in the perfused rat lungs (Fig. 3). Low uptake in the isolated rat lung perfused with blood-free buffer implied that the affinity of anti-PECAM is not sufficient for the targeting (total volume of buffer in the perfusion system is 45 ml vs. ~6 ml of plasma in rats). Theoretically, affinity of antigen binding is an individual feature of anti-PECAM clones. To address this issue, we characterized several anti-PECAM clones in terms of binding to immobilized CD31 and pulmonary uptake. Our results indicate that *1*) an affinity of anti-PECAM clones binding to human CD31 varies from 5 to 250 nM; *2*) these clones display low pulmonary uptake in perfusion and modest uptake in vivo; *3*) after conjugation with SA, some anti-PECAM clones (e.g., MAb 62) display very high uptake in the perfused lungs (30–40% ID/g) and in vivo (15% ID/g); and, *4*) the magnitude of the effect of SA differs for individual clones, e.g., SA-biotinylated (b) MAb 164.4 conjugate displays an intermediate uptake in perfusion (10% ID/g) and low uptake in vivo (2% ID/g).

We attribute the effect of SA to formation of multivalent complexes possessing a high affinity for binding to PECAM. Variations of the effect of SA with individual anti-PECAM clones may be explained by several reasons. First, the effect of SA on behavior of individual anti-PECAM clones may depend on their initial affinity. However, a very similar effect on pulmonary uptake of b-MAb 62 (K_d 10 nM) and b-Ab Houston (K_d 250 nM) in perfusion indirectly argues against this explanation. We favor an alternative explanation that the size of SA-b-anti-PECAM conjugates may differ for each individual biotinylated MAb clone. Proteins, including Igs, have a different amount of lysine residues suitable for biotinylation. Our recent studies show that various biotinylated MAb clones form complexes with SA that vary in size from 50 nm to 50 μm and that the size of the resulting SA-b-MAb complexes represents an extremely important parameter of the targeting (Wiewrodt R and Muzykantov, unpublished observations). This aspect of immunotargeting is currently under investigation in one of our laboratories (Muzykantov). The present results indicate that formation of anti-PECAM-SA conjugates dramatically facilitates pulmonary targeting of anti-PECAM. It is conceivable to postulate that affinity of binding of multivalent SA-anti-PECAM to CD31 is higher than that of bivalent antibody, albeit at the present time we cannot exclude potential contribution of facilitated internalization. A direct comparison of the K_d for anti-PECAM and SA-anti-PECAM to CD31 represents a formidable

task because interpretation of the results of either classical (e.g., Scatchard linearization) or modern (e.g., BioCore plasmon resonance) analyses depends on a ligand size. However, chemical modification of anti-PECAM with cross-linkers produces multivalent high-affinity, internalizable anti-PECAM complexes useful as carriers for vascular immunotargeting of enzymes and genes to the pulmonary vasculature in intact animals (4, 35, 42).

CD90.1 (Thy-1.1) antigen is a cell-surface glycoprotein initially identified as a cell differentiation marker expressed in the thymus, lymphoid tissues, hemopoietic stem cells, and neurons (6, 24). Our data show that anti-CD90.1 MAbs accumulate in the spleen (18% ID/g tissue) after systemic injection. This result correlates well with the high level of Thy-1.1 antigen in this organ, likely because of a high concentration of Thy-1.1-expressing blood cells and their precursors (Fig. 1). Effective splenic targeting of anti-Thy-1.1 corroborates with the literature indicating that Thy-1.1 antibodies could be used for delivery of liposomes to lymphoid tissues (13, 27). However, endothelial targeting and pulmonary uptake of anti-Thy-1.1 have not been addressed in the literature; to our knowledge, this paper presents the first evidence of pulmonary accumulation of anti-Thy-1.1. The nature and potential applicability of anti-Thy-1.1 uptake in the lungs (so explicitly manifested in the perfused organ) is worthy of further in-depth investigation. The images of tissue immunostainings (Figs. 1 and 2) show that anti-Thy-1.1 binds to endothelial cells. This result corroborates a recent observation (34) and provides a plausible explanation for the pulmonary accumulation of anti-Thy-1.1. However, pulmonary vasculature is known to contain a significant amount of resident Thy-1.1-positive white blood cells (15). Therefore, both binding to endothelium and to resident white blood cells may contribute to the lung targeting. The applicability of antibodies to Thy-1.1 for vascular immunotargeting must be addressed carefully, especially in the light of potential side effects on Thy-1.1 on the lymphoid and renal cells (45).

Mouse anti-rat endothelial MAb MRC OX-43 accumulates in the perfused rat lungs (Fig. 3). This indicates that the luminal surface of vascular endothelium expresses the antigen recognized by MRC OX-43 and that the affinity of the antibody is sufficient for binding. This MAb recognizes the endothelium of arteries, veins, and capillaries (except brain capillaries). In spleen and in the other organs, significant numbers of interstitial cells are labeled (Figs. 1*D* and 2*E*; also see Ref. 54). After in vivo injection, this MAb shows no detectable pulmonary uptake but accumulates in the spleen (7% ID/g tissue) and in the heart (2% ID/g), findings that correlate with immunohistochemical data. Although cardiac accumulation (a rare feature for carrier MAbs) is of interest, the lack of significant pulmonary targeting, as well as the unknown nature of the antigen and its function, limits speculation on potential applicability of MAb MRC OX-43.

In summary, our results support the paradigm that antigen accessibility to the bloodstream and antibody affinity represent more important determinants for the vascular immunotargeting than the total level of an antigen expression. This paradigm is shown in the perfused rat lung by a poor uptake of ^{125}I-anti-PECAM (despite extensive immunostaining) and high uptake of ^{125}I-anti-Thy-1.1 (despite relatively modest immunostaining). PECAM-1 is clearly localized in the vascular endothelium in the lung. Therefore, even a well-expressed endothelial antigen may provide a relatively poor targeting if an epitope is not accessible to the circulating antibody or if the antibody affinity does not suffice. In this context, means for augmentation of carrier affinity (such as SA modification) are of significant interest. In general, the tissue selectivity of MAb uptake correlates with that of an antigen presentation in the capillaries. It can be explained by the fact that capillaries represent the most extended endothelial target surface area in many organs. This paradigm is shown by the selective accumulation of anti-ACE MAb in the lungs, in good agreement with a high selectivity of ACE expression in the pulmonary capillaries. In fact, only the ACE antibody demonstrates a tissue-selective pulmonary uptake among the tested antibodies.

Results of this and other recent studies indicate that anti-ACE, anti-ICAM-1, and anti-PECAM-1 may serve as carriers for vascular immunotargeting, including delivery of drugs, enzymes, and genes to the pulmonary endothelium. Lung perfusion may permit even more effective and safe pulmonary targeting, potentially applicable in lung transplantation and cardiopulmonary bypass.

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Streptavidin facilitates internalization and pulmonary targeting of an anti-endothelial cell antibody (platelet-endothelial cell adhesion molecule 1): A strategy for vascular immunotargeting of drugs

(lung/bioconjugation/catalase/PECAM-1)

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ABSTRACT **Conjugation of drugs with antibodies to surface endothelial antigens is a potential strategy for drug delivery to endothelium. We studied antibodies to platelet-endothelial adhesion molecule 1 (PECAM-1, a stably expressed endothelial antigen) as carriers for vascular immunotargeting. Although ^{125}I-labeled anti-PECAM bound to endothelial cells in culture, the antibody was poorly internalized by the cells and accumulated poorly after intravenous administration in mice and rats. However, conjugation of biotinylated anti-PECAM (b-anti-PECAM) with streptavidin (SA) markedly stimulated uptake and internalization of anti-PECAM by endothelial cells and by cells expressing PECAM. In addition, conjugation with streptavidin markedly stimulated uptake of ^{125}I-labeled b-anti-PECAM in perfused rat lungs and in the lungs of intact animals after either intravenous or intraarterial injection. The antioxidant enzyme catalase conjugated with b-anti-PECAM/SA bound to endothelial cells in culture, entered the cells, escaped intracellular degradation, and protected the cells against H_2O_2-induced injury. Anti-PECAM/SA/^{125}I-catalase accumulated in the lungs after intravenous injection or in the perfused rat lungs and protected these lungs against H_2O_2-induced injury. Thus, modification of a poor carrier antibody with biotin and SA provides an approach for facilitation of antibody-mediated drug targeting. Anti-PECAM/SA is a promising candidate for vascular immunotargeting of bioactive drugs.**

Conjugation of drugs with monoclonal antibodies is a potential strategy for site-specific therapy (immunotargeting). Vascular immunotargeting has been proposed using antibodies to endothelial antigens, such as angiotensin-converting enzyme (ACE) (1), thrombomodulin (2), tumor endothelial antigens (3), E-selectin (4), and intercellular adhesion molecule-1 (ICAM-1)(5). Because the lungs contain ≈30% of endothelial cells in the body and receive the entire cardiac blood output, these antibodies accumulate in the lungs, providing targeting to the pulmonary endothelium (1, 2, 5).

None of the antibodies used to date for vascular immunotargeting has proven ideal. They all have relatively limited binding capacities [e.g., 1–3 × 10^5 binding sites per cell for anti-ACE (6)]. Poor internalization of anti-intercellular adhesion molecule by endothelium precludes intracellular delivery of a drug or genetic material (7). Although endothelium internalizes anti-thrombomodulin, it undergoes rapid cellular degradation (8). Anti-ACE selectively delivers drugs to the pulmonary endothelium and is internalized without rapid degradation (1, 5, 6). However, cytokines and oxidants suppress ACE expression in endothelium and, thus, inflammation may compromise targeting (9).

To develop a more efficient and safer strategy for drug targeting to endothelium, we studied antibodies to platelet-endothelial cell adhesion molecule-1 (PECAM-1, or CD31). PECAM-1 is a transmembrane adhesion molecule expressed at high levels on endothelial cells (≈1 million copies per cell) that plays an important role in transendothelial migration of leukocytes (10–12). Unlike ACE, expression levels are not markedly altered by cytokines (11).

We therefore evaluated the targeting profile of anti-PECAM in cell cultures and in animals. Although endothelial cells possessed very high anti-PECAM binding capacity, they did not internalize anti-PECAM. Similarly, although anti-PECAM intensely stained endothelium in the lung tissue, ^{125}I-labeled anti-PECAM accumulated poorly in the lungs.

However, because immunotargeting requires that the drug be coupled to a carrier, we studied the effect of conjugation of biotinylated anti-PECAM (b-anti-PECAM) with streptavidin (SA) on the targeting profile of b-anti-PECAM. In this paper, we report that streptavidin markedly facilitates internalization and pulmonary targeting of anti-PECAM and, therefore, provides a useful mechanism for delivery of drugs to endothelial cells. The effect of SA is not limited to anti-PECAM and endothelial cells. Therefore, SA-mediated conversion of poor carriers to highly internalizable/targetable carriers provides a paradigm for drug targeting strategies.

MATERIALS AND METHODS

Antibodies. Four PECAM-1 antibodies were used: (*i*) "Houston", a polyclonal rabbit IgG against human and rat PECAM-1 (13); (*ii*) mAb 62, provided by M. Nakada (Centocor) an IgG2 mAb reacting with human and rat PECAM-1 (14); (*iii*) mAb 4G6, a mouse mAb IgG2b reacting with the most membrane-proximal, sixth Ig-like loop of human PE-

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: SA, streptavidin; PECAM, platelet-endothelial cell adhesion molecule; b-anti-PECAM, biotinylated anti-PECAM; ACE, angiotensin-converting enzyme; HUVEC, human umbilical vein endothelial cells; IPL, isolated perfused lungs; ID/g, injected dose per gram; KRB, Krebs–Ringer buffer.

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CAM-1 (15); and (*iv*) mAb 390, a rat mAb reacting with murine PECAM-1/CD31 (15). Soluble purified PECAM was generously provided by P. Newman (Blood Center of Southeastern Wisconsin). Murine mAb 1045 recognizes a chondroitin sulfate-dependent epitope of human thrombomodulin (8).

Biotinylation, Radiolabeling, and Conjugation of Proteins. Proteins were biotinylated with 6-biotinylaminohexanoic acid *N*-hydroxysuccinimide ester (BxNHS, Calbiochem) without detectable reduction of functional activity and designated as b-IgG, b-mAb, or b-catalase (Fluka) following procedures described (16). Proteins were labeled with ^{125}I (Amersham Pharmacia) using Iodogen (Pierce). Catalase was conjugated with b-IgG or b-anti-PECAM by using a procedure described previously (16).

Binding, Internalization, and Degradation of ^{125}I-Labeled Anti-PECAM in Cell Culture. The following cell lines were used: (*i*) human umbilical vein endothelial cells (HUVEC); (*ii*) EAhy$_{926}$ cells (immortalized PECAM-expressing transformed hybrid cells), kindly provided by C. Edgel (University of North Carolina, Chapel Hill, NC) (17); and (*iii*) human mesothelioma REN cells or REN cells transfected with human PECAM-1 cDNA (REN/PECAM cells) (14). ^{125}I-labeled antibodies or conjugates were incubated with washed cells in M199 medium containing 0.2% BSA at 4°C or 37°C. After washing with M199, cells were incubated for 15 min with 50 mM glycine/100 mM NaCl, pH 2.5 to release surface-associated antibody. There was no detectable cell detachment or plasma membrane damage (determined by leakage of ^{51}Cr) immediately after treatment with glycine buffer. After glycine elution, cells were lysed with 0.1% Triton X-100. Cellular uptake was calculated as a sum of ^{125}I in the glycine eluates (surface-associated label) and the cell lysates (internalized label). Percent internalization was calculated as % = (total uptake − surface associated) × 100/(total uptake). To determine degradation of the ^{125}I-labeled proteins and detachment of ^{125}I from the proteins, cell lysates were incubated for 1 h at 4°C with 10% trichloroacetic acid and centrifuged, and ^{125}I in the pellet and in the supernatants was determined. Percent degradation was calculated as % = (supernatant ^{125}I) × 100/(supernatant ^{125}I + pellet ^{125}I) (6, 8).

Electron Microscopy of the Endothelial Uptake of b-Anti-PECAM/SA/b-Ferritin Complex. Confluent HUVEC (passage 2–3) grown on gelatin-coated 60 mm Petri dishes were incubated with b-anti-PECAM/SA/b-ferritin or b-IgG/SA/b-ferritin for 60 or 120 min at 37°C in M199 medium supplemented with 10% fetal bovine serum. After washing, cells were incubated for 10 min with ice-cold 2% paraformaldehyde/0.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) and processed for routine transmission electron microscopy.

Experiments in Isolated Perfused Rat Lungs. Preparation of isolated perfused lungs (IPL) was performed in anesthetized Sprague–Dawley male rats (170–200 g) as described (5, 6). IPL were ventilated with a humidified gas mixture containing 5% CO_2 and 95% air and perfused via the pulmonary artery with 45 ml of recirculating filtered Krebs–Ringer buffer (KRB) (pH 7.4), containing 10 mM glucose and 3% fatty-acid-free BSA at 37°C. ^{125}I-labeled proteins were perfused in IPL for 1 hr followed by 5 min with perfusion with KRB/BSA to eliminate nonbound material. ^{125}I uptake in the lungs was measured in a γ counter and expressed as a % of injected dose per gram (ID/g) (5). Catalase protection experiments were performed as described (5) by perfusing IPL for 1 hr with 45 ml of KRB/BSA containing 100 μg of catalase conjugated with either mouse IgG (IgG/Cat) or anti-PECAM mAb 62 (anti-PECAM/Cat). Control lungs were perfused with KRB/BSA. The nonbound material was eliminated by nonrecirculating perfusion of KRB/BSA for 5 min, and lungs were again perfused in a recirculating manner for 1 hr with KRB/BSA containing 5 mM H_2O_2. Lungs were rinsed with saline and gently blotted with a filter paper, and wet weight was determined. Thereafter lungs were dried and weighed to determine wet/dry lung weight coefficient.

***In Vivo* Injection of Radiolabeled Antibodies and Conjugates.** Biodistribution of ^{125}I-labeled antibodies and conjugates in intact animals was studied as described (1). One hour after injection of ^{125}I-labeled preparations into the tail vein in anesthetized Sprague–Dawley rats or BALB/c male mice, animals were sacrificed, internal organs were harvested and rinsed with saline, and the ^{125}I in tissues and in blood was determined in a γ counter.

RESULTS

Streptavidin Facilitates Intracellular Uptake of b-Anti-PECAM-1 Antibodies. HUVEC, EAhy$_{926}$, and REN/PECAM cells expressed PECAM-1 predominantly at the intercellular junctions (Fig. 1*A*) and possessed a very high binding capacity for ^{125}I-anti-PECAM. In HUVEC, Scatchard analysis revealed that K_d was 10 nM for mAb 62 (B_{max} = 1.5 × 10^5 per cell) and 250–350 nM for the polyclonal Ab Houston (B_{max} = 5–8 × 10^6 per cell). Neither biotinylation nor conjugation with SA altered the K_d of [^{125}I]anti-PECAM binding to HUVEC or REN/PECAM cells at 4°C (data not shown).

Despite high binding levels, a number of lines of evidence showed that PECAM-expressing cells internalized very small amounts (<10%) of bound anti-PECAM. First, binding of ^{125}I-anti-PECAM to cells at 37°C did not exceed that at 4°C. For example, in REN/PECAM cells, uptake of ^{125}I-mAb 62 was 6.9 ± 0.4 ng per well at 4°C vs. 6.0 ± 0.1 ng per well at 37°C. Second, glycine buffer eluted 81 ± 12% of ^{125}I from cells after

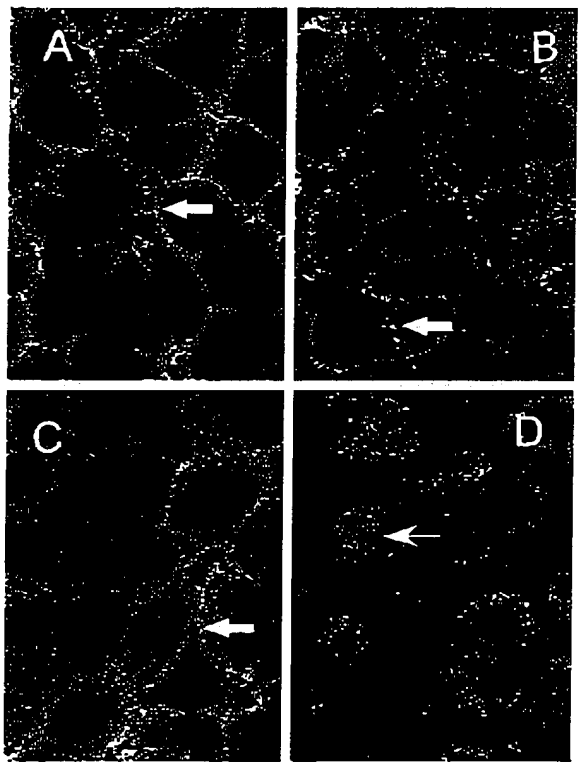

FIG. 1. SA facilitates internalization of biotinylated anti-PECAM. EAhy926 cells were incubated for 90 min with either b-Ab Houston (*A* and *B*) or b-Ab Houston conjugated with streptavidin (*C* and *D*) at 4°C (*A* and *C*) or at 37°C (*B* and *D*). After washing, fixation, and permeabilization, cells were stained with fluorescein isothiocyanate-labeled antibody against rabbit IgG. Note granular intracellular fluorescence in the cells incubated with b-anti-PECAM/SA (*D*, arrow); in *A*, *B*, and *C* the fluorescent signal is localized on the cellular surface at cell–cell borders (arrows).

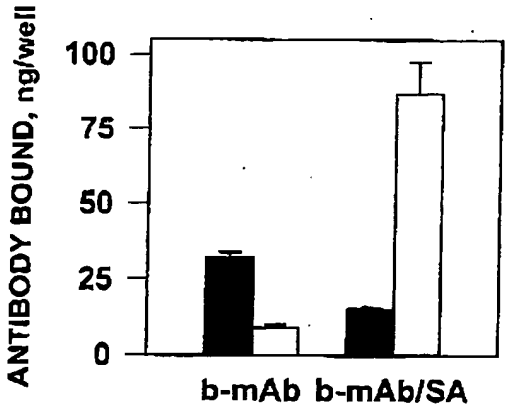

FIG. 2. SA stimulates the cellular uptake of biotinylated ^{125}I-anti-PECAM. REN/PECAM cells were incubated for 90 min at 37°C with ^{125}I-b-mAb 62 or ^{125}I-b-mAb 62/SA. After washing, ^{125}I in the surface fraction (filled bars) and cell lysates (open bars) in the cells was determined (mean ± SD, n = 3).

90 min of incubation with ^{125}I-mAb 62 at 37°C, a value almost identical to ^{125}I eluted after 90 min of incubation with ^{125}I-mAb 62 at 4°C (84 ± 15%). Third, anti-PECAM mAb incubated with cells for 90 min at either 37°C or 4°C showed clear localization to the plasma membrane with minimal cytoplasmic staining (Fig. 1 *A* and *B*). Poor internalization was seen with all PECAM antibodies in cell types tested. Biotinylation did not alter internalization of anti-PECAM by PECAM-expressing cells (data not shown).

In contrast, SA markedly stimulated the uptake at 37°C of b-^{125}I-mAb 62 by REN/PECAM cells (from 42 ± 2 to 102 ± 11 ng per well) and by HUVEC (from 16.2 ± 0.5 to 114.1 ± 5.3 ng per well) and stimulated the uptake of b-^{125}I-Ab Houston by HUVEC (data not shown). SA had no effect on the uptake of b-^{125}I-IgG by cells (e.g., 0.62 ± 0.1 and 0.61 ± 0.1 ng per well for b-^{125}I-IgG and b-^{125}I-IgG/SA in EAhy$_{926}$ cells).

SA also markedly facilitated b-^{125}I-anti-PECAM internalization by the target cells. In REN/PECAM cells, only 13 ± 10% of cell-bound b-^{125}I-mAb 62/SA was accessible for elution by glycine buffer (Fig. 2), indicating that 85–90% of antibody was internalized. SA caused 85–95% internalization of b-^{125}I-mAb 62 by HUVEC, and 80–95% internalization of b-^{125}I-mAb 4G6 and b-^{125}I-Ab Houston in all PECAM-expressing cells. SA had no effect on internalization of nonbiotinylated anti-PECAM, nor did b-^{125}I-IgG (data not shown).

Fig. 1 shows confocal microscope images of EAhy$_{926}$ cells incubated for 90 min with b-Ab Houston and b-Ab Houston/SA conjugate at 4°C or 37°C. Staining of the fixed permeabilized cells with fluorescein isothiocyanate-labeled secondary antibody revealed surface (cell–cell) localization of both b-anti-PECAM and b-anti-PECAM/SA at 4°C. Although cellular distribution of b-anti-PECAM at 37°C did not differ markedly from that at 4°C, b-anti-PECAM/SA accumulated intracellularly at 37°C. Similar results were reproduced in HUVEC and in REN/PECAM cells with b-mAb 62 and Ab Houston (data not shown).

Fig. 3 shows transmission electron microscopic images of the cellular uptake of b-ferritin conjugated with b-mAb 62/SA or with b-IgG/SA. After 1 hr of incubation at 37°C, anti-PECAM/SA/ferritin was localized predominantly in the sites of intercellular contacts (Fig. 3*D*) and in the invaginations of the plasma membrane packed with the conjugate (Fig. 3*E*).

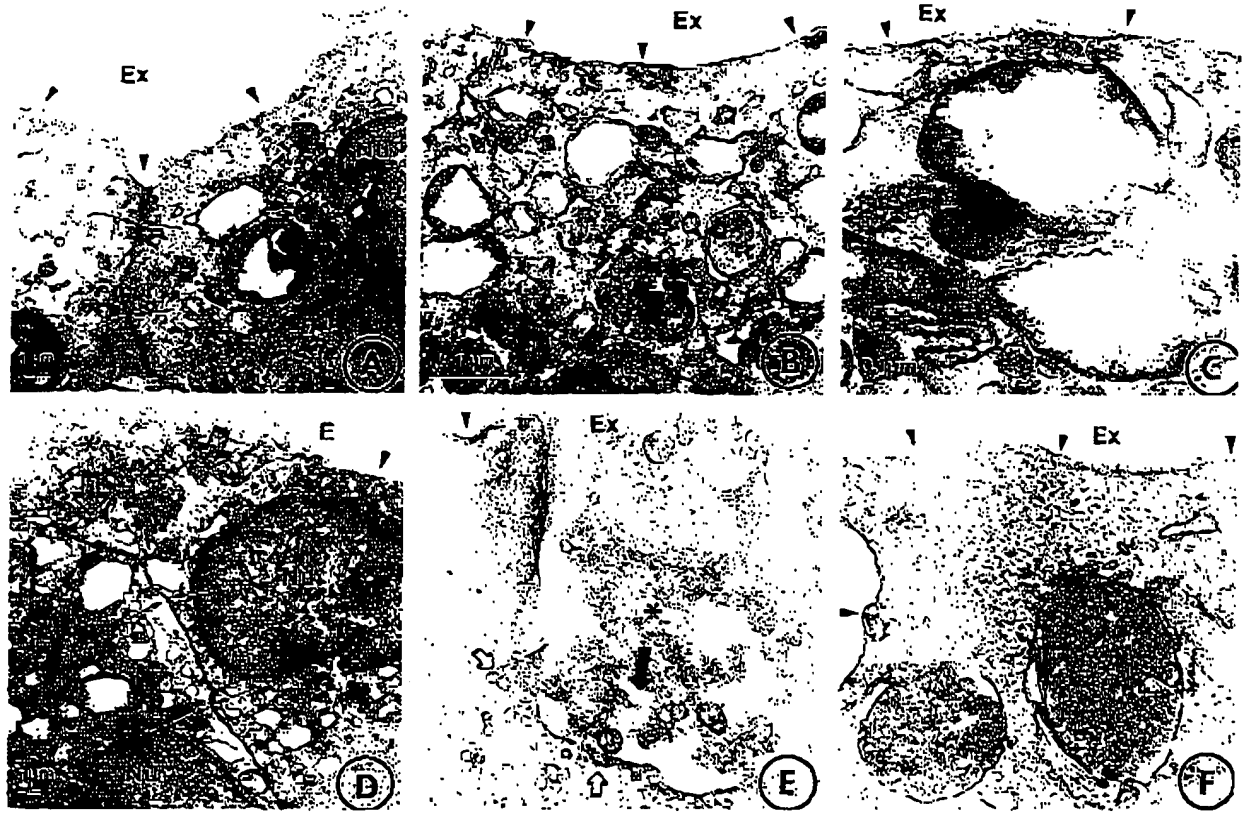

FIG. 3. Uptake of b-ferritin conjugated with b-mAb 62/SA by endothelial cells. HUVEC were incubated with b-IgG/SA/b-ferritin (*A–C*) or b-mAb 62/SA/b-ferritin (*D–F*) for 60 min (*A*, *B*, *D*, and *E*) or 120 min (*C* and *F*) at 37°C, fixed, and processed for electron microscopy. *, ferritin; arrowheads, the plasma membrane; Nu, nucleus; Ex, extracellular space; small arrows in *A* and *D*, the intracellular contact; large open arrows in *D*, cell-associated ferritin conjugate; large closed arrow in *E*, direction of the conjugate uptake; small open arrows in *E*, microvesicles associated with large invagination of the plasma membrane.

After 2 hr of incubation at 37°C, the conjugate was found predominantly in large intracellular vacuoles (Fig. 3*F*), whereas the plasma membrane appeared to be cleared of the conjugate. Fig. 3*A–C* show that there was no detectable uptake of IgG/SA/ferritin.

Streptavidin did not alter the rate of b-anti-PECAM degradation by cells. For example, only 1.9 ± 0.7% or 0.7 ± 0.2% of trichloroacetic acid-soluble ^{125}I was detected in cell lysates after incubation of b-^{125}I-mAb 62 or b-^{125}I-mAb 62/SA with REN/PECAM cells for 90 min at 37°C. Trichloroacetic acid-soluble ^{125}I in the cell medium after incubation did not exceed 2% in either case. Similar results were obtained in HUVEC (data not shown). Thus, the rate of cellular degradation of b-^{125}I-mAb 62/SA was slow, despite its effective internalization.

The Effect of SA on Internalization of Biotinylated Antibodies Is Generalizable and Does Not Depend on the Intracellular Domain of PECAM or on the RGD Domain of SA. We also studied the effect of SA on internalization of b-mAb 1045, an antibody against thrombomodulin that is poorly internalized by HUVEC (8). Like our results with PECAM-1, we found that SA stimulated internalization from 20.5 ± 8.3% for nonconjugated b-^{125}I-mAb 1045 to 92 ± 7% for b-^{125}I-mAb 1045/SA. This result implies that the effect of SA is not specific for PECAM-1.

To determine whether the PECAM-1 intracellular domain was involved in the internalization of anti-PECAM/SA, we compared the uptake and internalization of b-^{125}I-mAb 62/SA by REN cells transfected with full-length PECAM-1 or a truncated mutant form of PECAM-1 lacking the intracellular domain. Internalization of ^{125}I-anti-PECAM/SA was equally effective in both cell types (≈95%), indicating that the intracellular domain of PECAM-1 is not important for the SA-facilitated uptake of anti-PECAM.

We compared the effects of SA, a neutral tetrameric biotin-binding protein possessing an RGD-like sequence (RYD), and neutravidin, a neutral derivative of the tetrameric biotin-binding protein avidin that lacks its RYD domain. Both proteins stimulated uptake and binding of ^{125}I-anti-PECAM to a similar extent (data not shown), thus indicating that the RYD domain of SA is not important for the SA-facilitated intracellular delivery of anti-PECAM.

To determine whether binding/uptake of anti-PECAM/SA conjugate activated nonspecific fluid-phase uptake of proteins, we incubated REN/PECAM cells with ^{125}I-albumin in the presence or absence of nonlabeled anti-PECAM/SA for 90 min at 37°C. In control cells, cellular uptake of ^{125}I-albumin was 104 ± 19 pg per well, with internalization of 69 ± 20% of ^{125}I. Neither anti-PECAM/SA nor IgG/SA stimulated cellular uptake (96 ± 18 and 89 ± 6 pg per well) or internalization of ^{125}I-albumin (44 ± 9% and 62 ± 17%).

SA Facilitates Targeting of Biotinylated Anti-PECAM to the Pulmonary Vascular Endothelium. In rat lungs stained with anti-PECAM-1 mAb 62, we found intense staining of endothelium in the large and small vessels (data not shown), in good agreement with our previous results with the polyclonal antibody Houston (12). We next determined the pulmonary uptake of ^{125}I-anti-PECAM in rat IPL. Although pulmonary uptake of ^{125}I-mAb 62 in the IPL was 5-fold higher than that of control ^{125}I-IgG (2.3 ± 0.2 vs. 0.5 ± 0.2% of ID/g), uptake of ^{125}I-anti-PECAM in the IPL was an order of magnitude lower than that of ^{125}I-anti-ACE (21 ± 3% ID/g). In intact rat experiments, ^{125}I-mAb 62 displayed no specific pulmonary targeting after injection (3.6 ± 1.1 vs. 4.3 ± 0.7% ID/g for ^{125}I-IgG). Thus, despite high levels of PECAM expression in the pulmonary vasculature, anti-PECAM displayed poor targeting potential.

Because SA stimulated cellular binding and internalization of b-anti-PECAM in cell culture, we studied the pulmonary uptake of b-^{125}I-anti-PECAM/SA in intact animals. In contrast to the low uptake of unconjugated b-^{125}I-mAb 62 (3.6 ± 1.1% ID/g), pulmonary uptake was 14.2 ± 3.0% ID/g 1 hr after i.v. injection of b-^{125}I-mAb 62/SA in rats (Fig. 4*A*). Facilitation of the targeting of b-anti-PECAM was specific, because SA reduced pulmonary uptake of b-^{125}I-IgG (2.1 ± 1.1% for b-^{125}I-IgG/SA vs. 4.3 ± 0.7% ID/g for b-^{125}I-IgG). SA conjugation reduced the blood levels of both b-IgG and b-mAb 62 in rats. Thus, the lung/blood ratio of b-^{125}I-mAb 62/SA was 25.9 ± 4.8, vs. 1.3 ± 0.6 for b-^{125}I-IgG/SA. The stimulation of pulmonary uptake was reproduced in mice by using the rat anti-mouse PECAM b-mAb 390: the pulmonary uptake of b-^{125}I-mAb 390/SA was 30 ± 6% ID/g vs. 4.9 ± 0.4% ID/g for b-^{125}I-IgG/SA. SA reduced the pulmonary level of b-^{125}I-IgG by 50% in mice, thus confirming the specificity of anti-PECAM/SA targeting.

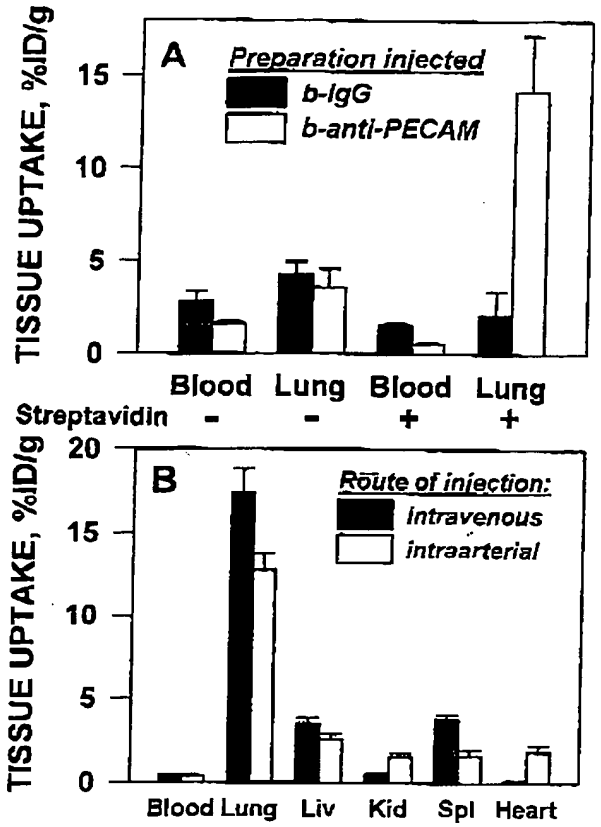

FIG. 4. Pulmonary targeting of b-^{125}I-anti-PECAM/SA *in vivo*. (*A*) Rats were injected via tail vein with ^{125}I-b-IgG (filled bars) or ^{125}I-b-anti-PECAM (open bars) either conjugated with streptavidin (+) or without streptavidin (−). (*B*) Rats were injected with b-^{125}I-anti-PECAM/SA via the tail vein (filled bars) or via the left ventricle (open bars). One hour after injection, rats were sacrificed, and ^{125}I in the tissues was determined (mean ± SD, n = 5).

To exclude the role of blood in the pulmonary targeting, we tested the uptake of b-anti-PECAM/SA in IPL perfused with blood-free buffer. SA caused 20-fold stimulation of the pulmonary uptake of b-^{125}I-mAb 62 (39 ± 3.1% ID/g) and b-^{125}I-Ab Houston (53.1 ± 9.35 ID/g) with no effect on b-^{125}I-IgG (0.6+0.1% ID/g) or nonbiotinylated ^{125}I-anti-PECAM (2.3+0.3% ID/g). Both in IPL and in intact rats, neutravidin stimulated pulmonary uptake of b-^{125}I-mAb 62 to the same extent as SA (data not shown).

Fig. 4*B* shows the distribution of b-^{125}I-mAb 62/SA after intravenous or intraarterial injection in rats. Both routes of administration provided high pulmonary uptake of the conjugate, implying that pulmonary targeting is not caused by mechanical retention of anti-PECAM/SA in capillaries. Because the injected dose (less than 1 μg/kg) was below satura-

tion, a first passage phenomenon contributed to the conjugate distribution in the organs. Thus, intraarterially injected b-^{125}I-mAb 62/SA displayed higher cardiac and renal uptake and lower pulmonary, hepatic, and splenic uptake.

Anti-PECAM/SA/b-Catalase Accumulates Intracellularly in Endothelial Cells and Protects from H_2O_2-Mediated Injury in Cell Culture. To evaluate b-anti-PECAM/SA as a carrier for endothelial targeting of drugs, we conjugated it with b-catalase and studied the uptake and activity of b-anti-PECAM/SA/b-catalase *in vitro*. The b-mAb 62/SA/b-catalase, but not b-IgG/SA/b-catalase, specifically bound in the PECAM-coated wells (Fig. 5) and degraded H_2O_2 in the wells (Fig. 5 *Inset*). Whereas b-IgG/SA/b-^{125}I-catalase did not bind to HUVEC (1.3 ± 0.1 ng per well), both b-mAb 62/SA or b-Ab Houston/SA provided high-level binding of b-^{125}I-catalase (67.2 ± 2.9 and 184.5 ± 0.3 ng per well). HUVEC internalized >90% of b-anti-PECAM/SA/b-^{125}I-catalase, whereas degradation of internalized catalase did not exceed 5%. The antibody-conjugated catalase was not degraded and then secreted, because only 5.6 ± 1.2% of TCA-soluble ^{125}I was detected in the cell medium after 90 min of incubation of b-mAb 62/SA/b-^{125}I-catalase at 37°C in HUVEC. In REN/PECAM cells, the intracellular uptake of anti-PECAM/^{125}I-catalase was 195 ± 8 ng per well (vs. 3.5 ± 0.5 ng per well for IgG/catalase), with 95% internalization of the cell-bound ^{125}I-catalase. In EAhy$_{926}$ cells, internalization of b-Ab Houston/SA/b-^{125}I-catalase was 78.1 ± 3.3%.

To determine whether the conjugate could protect cultured cells from H_2O_2-induced injury, HUVEC were labeled with ^{51}Cr, incubated for 1 hr at 37° with b-mAb 62/SA/b-catalase or b-IgG/SA/b-catalase, washed, and exposed to 2 mM H_2O_2 for 6 hr. H_2O_2 induced 14.5 ± 1.3% and 15.1 ± 1.3% release of ^{51}Cr from HUVEC treated with either b-mAb 62/SA/b-catalase or b-Ab Houston/SA/b-catalase, vs. 56.1 ± 5.7% of ^{51}Cr released from cells treated with b-IgG/SA/b-catalase. In control cells, H_2O_2 caused 73 ± 33% release of ^{51}Cr. After subtraction of the level of spontaneous ^{51}Cr release from control cells not treated with H_2O_2 (10.9 ± 0.6%), anti-PECAM/catalase afforded a 91% reduction of ^{51}Cr release. Thus, anti-PECAM/SA/b-catalase protected cells against oxidative injury by H_2O_2, whereas b-IgG/SA/b-catalase provided no significant protection.

Anti-PECAM/SA/b-Catalase Accumulates in the Lungs and Protects from H_2O_2-Mediated Injury in the Lung. To test the ability of anti-PECAM/SA to deliver an active drug to the pulmonary endothelium, b-anti-PECAM/SA/b-^{125}I-catalase or b-IgG/SA/b-^{125}I-catalase were injected intravenously into intact animals. In rats, b-mAb 62/SA/b-^{125}I-catalase specifically accumulated in rat lungs after intravenous injection, with lung/blood ratio in rats 39.8 ± 4.1 for b-mAb 62/SA/b-^{125}I-catalase vs. 1.1 ± 0.2 for b-IgG/SA/b-^{125}I-catalase (Fig. 6). Similar results were seen in mice, with lung/blood ratio equal to 7.5 ± 1.1 for b-mAb 390/SA/b-^{125}I-catalase vs. 0.6 ± 0.1 for b-IgG/SA/b-^{125}I-catalase.

Finally, based on these results, we examined the ability of b-mAb 62/SA/b-catalase to protect the lung against intravascular oxidative insult. In the first experiment, we determined that the uptake of b-mAb 62/SA/b-^{125}I-catalase and b-IgG/SA/b-^{125}I-catalase in IPL was 37.3 ± 4.4% vs. 2.1 ± 0.2% ID/g (1-hr perfusion). In the second experiment, we used perfusion of 5 mM H_2O_2 in IPL, an intervention that causes lung injury causing elevation of the lung wet/dry ratio, reflecting lung edema (5). IPL were first perfused for 1 hr with 100 μg of b-mAb 62/SA/b-catalase, b-IgG/SA/b-catalase, or buffer alone. After elimination of nonbound material, lungs were further perfused with 5 mM H_2O_2 for 60 min. In IPL treated with b-IgG/SA/b-catalase, the wet/dry weight ratio (8.1 ± 0.7) was markedly higher ($P < 0.001$) than that in the control lungs not treated with H_2O_2 (5.1 ± 0.2), indicating lack of protection against H_2O_2. In contrast, in IPL treated with b-mAb 62/SA/b-catalase, the wet/dry weight ratio remained normal (5.5 ± 0.1), thus indicating protection of the lung against H_2O_2-induced oxidative vascular injury.

DISCUSSION

The two major goals of most drug delivery strategies are recognition of specific cells in the body and effective intracellular delivery. Our results demonstrate that SA conjugation to biotinylated antibodies can convert antibodies with relatively poor targeting potential to very effective carriers for vascular immunotargeting.

SA, avidin, and its derivative, neutravidin, are tetrameric 60- to 66-kDa proteins possessing four high-affinity binding sites for biotin or biotinylated compounds (18). The SA-biotin crosslinking pair is useful for chemical conjugation of biomolecules. SA is nontoxic and does not cause harmful reactions in laboratory animals or in humans (19). SA-biotin crosslinkers have recently been used *in vivo* for targeting of drugs, toxins, radiolabels, and genetic material (19–21). It has been noted that biotinylation may reduce the affinity of antibodies (22)

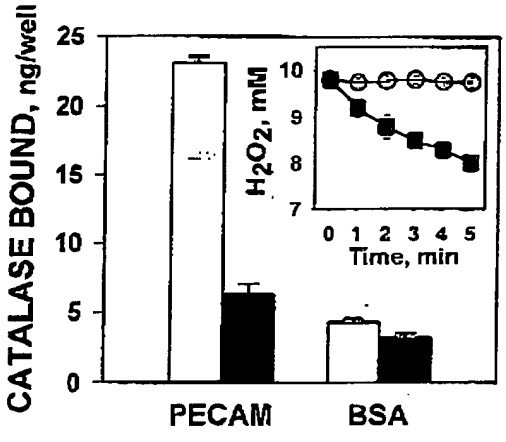

FIG. 5. Functional activity of b-anti-PECAM/SA/b-catalase complex. Ten micrograms of b-mAb 62/SA/b-^{125}I-catalase (open bars) or b-IgG/SA/b-^{125}I-catalase (filled bars) were incubated for 1 hr in wells coated with PECAM-1 or BSA. After washing, ^{125}I in the wells was determined. (*Inset*) Wells coated with BSA (○) or PECAM (■) were incubated with 20 μg of b-mAb 62/SA/b-catalase for 1 hr. After washing, 10 mM H_2O_2 was added to the wells and its decay was determined.

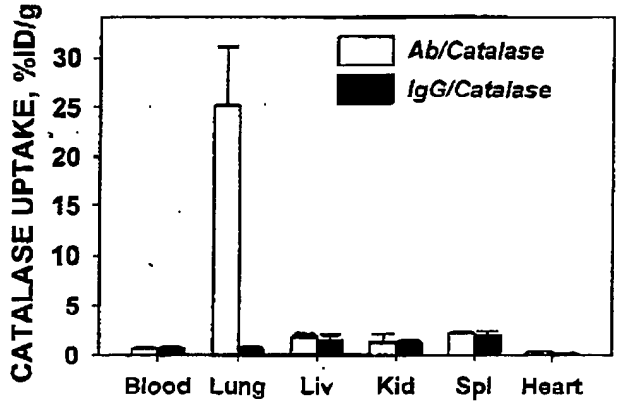

FIG. 6. Pulmonary targeting of b-anti-PECAM/SA/b-^{125}I-catalase in rats. Ten micrograms of b-anti-PECAM/SA/b-^{125}I-catalase (open bars) or b-IgG/SA/b-^{125}I-catalase (filled bars) was injected in the tail vein of anesthetized rats. One hour later, rats were sacrificed, and ^{125}I in the tissues was determined (mean ± SD, n = 3–5).

and that SA accclerates elimination of biotinylated antibodies from the bloodstream (23) and may hinder interaction of biotinylated enzymes with their substrates (16). In the present study, we did not observe changes in the biological behavior of b-anti-PECAM with regard to binding to immobilized antigen or uptake by PECAM-expressing cells. However, we found that SA caused dramatic stimulation of cellular binding, internalization, and pulmonary targeting of biotinylated antibodies.

The pulmonary targeting cannot be explained by mechanical retention of large complexes in the pulmonary capillaries because: (*i*) b-IgG/SA conjugates do not accumulate in the lungs; (*ii*) SA stimulates specific uptake of b-anti-PECAM in cell cultures; and (*iii*) both intravenous and intraarterial administration of b-anti-PECAM/SA provide pulmonary targeting. Thus, facilitation of the cellular uptake (i.e., binding and internalization) of b-anti-PECAM by SA is likely the mechanism for the pulmonary targeting. Because PECAM is expressed by normal endothelium in various vascular areas, an anti-PECAM/SA carrier may be useful for drug delivery to endothelium in various organs, depending on the route and mode of administration.

Importantly, conjugates escaped massive intracellular degradation, thus allowing the successful intracellular delivery of an active antioxidant enzyme, catalase, in our cell culture models. Our animal studies confirm the potential therapeutic applicability of this approach, although the final (intra)cellular destination of the conjugates in animal models needs to be addressed more precisely. The strategy of targeting of enzymes such as catalase or superoxide dismutase may be useful for the cell-specific augmentation of antioxidant defense in the pulmonary endothelium and protection against oxidative insults such as the adult respiratory distress syndrome or other disease conditions associated with or manifested by oxidative endothelial injury (24).

The exact mechanisms of how SA affects the targeting, cellular uptake, and intracellular trafficking of biotinylated anti-PECAM have yet to be determined. Neither the cytoplasmic domain of PECAM nor an RGD-like domain on SA plays an important role in cellular uptake of b-anti-PECAM/SA. Preliminary studies suggest that the size of the b-anti-PECAM/SA complex is an important determinant for the cellular uptake, and we favor a mechanism that involves a phagocyte-like uptake of complexes in large vacuoles (see Fig. 3*F*) in which proteins seem to be protected, at least initially, from rapid degradation.

The targeting strategy we have described here will likely be applicable to a wide range of therapeutic or experimental compounds and agents. For example, in addition to delivery of antioxidant enzymes, we have found that conjugation of biotinylated glucose oxidase to anti-PECAM/SA provides specific targeting of active biotinylated glucose oxidase to PECAM-1-expressing cells resulting in intracellular generation of H_2O_2 and severe oxidative stress in endothelial cells in culture, in perfused rat lungs, and in intact mice. Another potential use of the strategy described in the present paper could be vascular gene therapy. In preliminary experiments, we have found that b-anti-PECAM/SA/DNA conjugates specifically bind to REN/PECAM cells and result in 90% internalization of radiolabeled DNA, allowing transfection of the target cells.

In the wider context of general strategies for drug delivery, our results suggest that SA conjugation of an otherwise poorly internalizable/targetable antibody could convert it to a highly internalizable/targetable carrier. This observation thus serves as a paradigm for a novel drug targeting strategy. Although we have focused on the anti-PECAM antibodies in this study, SA-mediated internalization of biotinylated mAb 1045 against thrombomodulin indicates that this effect is not limited to PECAM antibodies. Moreover, effective internalization of anti-PECAM/SA by nonendothelial cells transfected with PECAM (REN mesothelioma cells) implies that the strategy described in the present paper may be applicable to a wide variety of target cells that could include tumor cells or HIV-infected cells.

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2

Characterization of Endothelial Internalization and Targeting of Antibody–Enzyme Conjugates in Cell Cultures and in Laboratory Animals

Silvia Muro, Vladimir R. Muzykantov, and Juan-Carlos Murciano

Summary

Streptavidin–biotin conjugates of enzymes with carrier antibodies provide a versatile means for targeting selected cellular populations in cell cultures and in vivo. Both specific delivery to cells and proper subcellular addressing of enzyme cargoes are important parameters of targeting. This chapter describes methodologies for evaluating the binding and internalization of labeled conjugates directed to endothelial surface adhesion molecules in cell cultures using anti-intercellular adhesion molecule/catalase or antiplatelet endothelial cell adhesion molecule/catalase conjugates as examples. It also describes protocols for characterization of biodistribution and pulmonary targeting of radiolabeled conjugates in rats using anti-intercellular adhesion molecule/tPA conjugates as an example. The experimental procedures, results, and notes provided may help in investigations of vascular immunotargeting of reporter, experimental, diagnostic, or therapeutic enzymes to endothelial and, perhaps, other cell types, both in vitro and in vivo.

Key Words: Endothelium; cell adhesion molecules; catalase; plasminogen activators; lung targeting.

1. Introduction

Streptavidin crosslinking of reporter and therapeutic enzymes with antibodies to endothelial cell adhesion molecules provides nanoscale conjugates useful for experimental and, perhaps, diagnostic or therapeutic vascular immunotargeting (*see* Chapter 1 and **refs. *1–3***). Binding and appropriate subcellular addressing of antibody–enzyme conjugates to and/or into the target cells are key components for optimal design of drug-delivery systems. The size of the conjugates is an important parameter that determines the rate of intracellular uptake and, perhaps, subcellular trafficking of the conjugates ***(4,5)***.

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This chapter outlines basic experimental protocols useful in the characterization of these relevant conjugates parameters. The first part (**Subheading 3.1.**) describes protocols for cell culture experiments that use fluorescent and radioisotope labeling as means to trace binding, internalization, and fate of anti-platelet endothelial cell adhesion molecule (PECAM)/catalase and anti-intercellular adhesion molecule (ICAM)/catalase conjugates. The second part (**Subheading 3.2.**) describes protocols for in vivo experiments in intact anesthetized rats using anti-ICAM/tissue-type plasminogen activator (tPA) conjugate labeled with radioisotopes. Thus, particular immunoconjugates described in this chapter are potentially useful for vascular targeting of either antioxidant (e.g., catalase to detoxify H_2O_2, **ref. *6***) or antithrombotic enzymes (e.g., tPA to dissolve fibrin, **ref. *3***). However, because of the modular nature of the conjugation and labeling procedures used, the described protocols can be used for the characterization of endothelial targeting and uptake of diverse reporter and therapeutic enzyme cargoes conjugated with a variety of carrier antibodies *(7)*. Furthermore, cell culture protocols given here for endothelial cells can be applied to other cell types of interest.

2. Materials

2.1. Equipment

1. Gamma-counter.
2. Fluorescence microscope equipped with ×40 or ×60 magnification objectives; filters compatible with fluorescein isothiocyanate (FITC; green), Texas Red (red), and UV or Alexa Fluor 350 (blue) fluorescence; digital camera; and image analysis software (ImagePro).

2.2. Reagents, Proteins, and Antibodies

1. Standard phosphate buffer, PBS, (NaH_2PO_4 20 m*M*, 150 m*M* NaCl, pH 7.4).
2. Glycine solution (50 m*M* glycine, 100 m*M* NaCl, pH 2.5) is used for elution of conjugates or antibodies bound to antigen expressed in the cells.
3. Lysis buffer (PBS containing 2% Triton X-100) is used to lyse cells and differentiate the internalized from the surface retained fractions of conjugates or antibodies.
4. PBS containing 5% bovine serum albumin (PBS-BSA) is used to increase the protein content in the analysis of free iodine label released from damaged proteins.
5. PBS containing 10% fetal bovine serum (PBS-FBS) is used to block the unspecific binding of conjugates or antibodies to the cells while providing the cell necessary nutrients.
6. Antibodies: human anti-ICAM-1 (MAb R6.5) or rat anti-ICAM-1 (MAb 1A29); human anti-PECAM-1 (MAb 62); and goat anti-mouse IgG conjugated to FITC, Texas Red, or Alexa Fluor 350.

7. Other reagents: Concentrated (100% w/v) trichloroacetic acid solution (TCA); goat serum; FITC-labeled streptavidin; tPA; catalase; paraformaldehyde; mowiol; [^{125}]iodine.

2.3. Immunoconjugates

^{125}I-labeled and nonlabeled immunoconjugates synthesized and characterized by dynamic light scattering as described in Chapter 1, where radioisotope is coupled to the cargo enzyme, not the carrier antibody, were used. In some cases, anti-ICAM/catalase conjugates based on FITC-labeled polymer were used *(4,5)*.

2.4. Cells and Media

1. Human umbilical vein endothelial cells (HUVECs, Clonetics).
2. Endothelial cell growth medium (*see* Chapter 1 for details on medium composition) free of antibiotics.

3. Methods

3.1. Characterization of Immunoconjugates in Cell Culture

3.1.1. Quantitative Tracing of Radiolabeled Conjugates in HUVECs

1. Seed the cells in 24-well plates. Cultivate to confluence (approx 48 h) in the appropriate medium. Replace by fresh antibiotic-free medium 24 h before the experiment.
2. Wash cells twice by warm (37°C) culture medium. Add 0.5 µg to 1 µg of conjugate per well (i.e., specific activity 0.03 µCi/µg to 0.1 µCi/µg) in 0.5 mL of medium supplemented with 10% FBS. Incubate cells for 1–2 h at 37°C in the presence of the immunoconjugates.
3. Wash cells three times by medium to remove nonbound conjugates. Incubate cells with a glycine solution (15 min, room temperature [RT]) to elute noninternalized immunoconjugates bound to the cell surface. Using a gamma-counter, determine radioactivity in glycine-eluted fraction (*see* **Note 5**).
4. Wash cells three times by medium and incubate them for 15 min at RT with 0.5 mL of lysis buffer. Add 0.1 mL of the obtained cell lysates to 0.5 mL of PBS-BSA and sequentially add 0.2 mL of TCA and incubate 20 min at RT to precipitate proteins. Centrifuge TCA–lysate mixture (3000*g*, 10 min) and determine radioactivity in pellet and supernatant fractions.
5. Determine protein concentration in a fraction of cell lysates to normalize radioactivity values in samples per gram of total cell protein. Relative and absolute binding, internalization, and/or degradation of the immunoconjugates can be calculated as follows:

$$\textit{Total binding} = \frac{\textit{cpm in glycine fraction} + \textit{cpm in lysate pellet fraction} + \textit{cpm in lysate supernatant fraction}}{\textit{specific activity (cpm/ng of conjugate)}}$$

$$\text{Internalization percentage (if applicable)} = 100 \times \frac{\text{cpm in lysate fraction}}{\text{cpm in lysate fraction + cpm in glycine-eluted fraction}}$$

$$\text{Total internalization (if applicable)} = \frac{\text{cpm in lysate fraction}}{\text{specific activity (cpm/ng of conjugate)}}$$

$$\text{Degradation percentage} = 100 \times \frac{\text{cpm in supernatant fraction}}{\text{cpm in glycine + cpm in lysate supernatant + cpm in lysate pellet fractions}}$$

$$\text{Total degradation} = \frac{\text{cpm supernatant fraction}}{\text{specific activity (cpm/ng of conjugate)}}$$

3.1.2. Subcellular Detection of Immunoconjugates by Immunofluorescence

3.1.2.1. Binding of Immunoconjugates to Target Cells

1. Seed the cells onto 12-mm^2 glass coverslips coated with 1% gelatin in 24-well plates. Allow cells to grow for 48 h to confluence (**Note 2**). Replace medium by fresh antibiotic-free medium 24 h before the experiment. Incubate cells for 5 min at 4°C before the experiment. Wash cells twice and replace by medium containing 10% FBS and a conjugate (1–1.5 µg of per well). Incubate cells for 30 min at 4°C to permit binding.
2. Wash cells three times with cold medium to eliminate nonbound conjugates. Fix cell by a cold solution 2% paraformaldehyde in PBS (15 min) (**Note 3**).
3. Wash cells three times with PBS and stain surface-bound conjugates by incubating fixed cells for 30 min at RT with a 4 µg/mL solution of Texas Red-labeled goat anti-mouse IgG in PBS-FBS (alternatively, use fluorescently labeled antibodies against the enzyme cargo) (**Note 1**). Wash cells three times with PBS.
4. Mount cell-containing coverslips on glass microscope slides using mowiol and incubate overnight at RT to allow the mounting media to polymerize. Observe samples by fluorescence microscopy using ×40 or ×60 objectives. Compare images of fluorescence and phase-contrast fields to confirm location of the immunoconjugate to the cell surface.

3.1.2.2. Internalization of Immunoconjugates Into Target Cells

1. Seed and grow cells to confluence as described in **Subheading 3.1.2.1.**
2. Wash cells twice with 37°C prewarmed medium and add immunoconjugate and incubate with cells for 1 h at 37°C to permit binding and internalization. Fix cells and stain surface-bound conjugates as described in **Subheading 3.1.2.1.**
3. Wash cells three times with PBS and permeabilize them by 15-min incubation with a cold solution 0.2% Triton X-100 in PBS. Stain internalized conjugates by incubating permeabilized cells with FITC-labeled goat anti-mouse IgG (4 µg/mL in PBS serum).
4. Wash cells and mount coverslips on microscope slides as described in **Subheading 3.1.2.1.**

5. Take images using filters compatible with Texas Red (red) and FITC (green) in a fluorescence microscope (×40 or ×60 objective) and merge them. Surface-bound conjugates will appear yellow (double-labeled), whereas internalized conjugates will be single-labeled in green. Imaging software can be programmed to quantify relative conjugate internalization, following the formula:

$$\text{Internalization percentage} = 100 \times \frac{(\text{number of green conjugates} - \text{number of red conjugates})}{\text{number of green conjugates}}$$

3.1.2.3. Fate of Intracellularly Delivered Immunoconjugates

1. For this type of experiments, use fluorescently labeled conjugates (i.e., based on FITC-labeled nondegradable polymer beads; *see* **Note 4**) prepared as previously described in detail *(4,5)*. First, incubate cells in the presence of conjugates at 4°C to permit binding to the cell surface. Then, wash nonbound immunoconjugates with cold medium, add FBS-supplemented medium, and incubate cells for the time period of interest at 37°C to permit endocytosis and intracellular trafficking of the immunoconjugates previously bound to the cell surface.
2. Wash and fix cells as in **Subheading 3.1.2.1.** followed by staining of the noninternalized conjugates for 30 min at RT with a solution 4 μg/mL goat anti-mouse IgG (i.e., labeled with Alexa Fluor 350) in PBS serum.
3. Wash the preparations three times with PBS and permeabilize cells for 15 min with a cold solution 0.2 % Triton X-100 in PBS. Incubate permeabilized cells with a solution 4 μg/mL goat anti-mouse IgG (i.e., labeled with Texas Red) in PBS serum.
4. Wash cells and mount coverslips on microscope slides as described in **Subheading 3.1.2.1.**

Inspect in a fluorescence microscope using filters compatible with FITC (green), Alexa Fluor 350 (blue), and Texas Red (red) and merge images. Immunoconjugates bound to the cell surface will appear triple-labeled as white. Nondegraded internalized conjugates will appear as double-labeled in yellow, whereas internalized counterparts with degraded protein component will be single-labeled as green.

3.2. Characterization of Immunoconjugates In Vivo

3.2.1. Biodistribution of Radiolabeled Conjugates After Intravenous Administration

1. Anesthetize rats (Sprague–Dawley) weighing 250 g using an intraperitoneal injection of 300 μL of Nembutal solution (70 mg/kg of body weight) and wait 5 min until animals are fully anesthetized (i.e., they do not react to their legs being squeezed with forceps).
2. Inject ^{125}I-labeled conjugates (approx 1–5 μg of the conjugate, 100,000–300,000 cpm per animal) via a tail vein in 0.2 mL of PBS using an insulin syringe with a

27.5-gage needle. Warming up the tail by using hot water makes the vein more visible and easy to inject.

3. One hour after injection, sacrifice anesthetized animals by dissection of the descending aorta, collect 1 mL of blood from the peritoneal cavity, and place it in a heparin-containing tube. Excise internal organs, including lung, liver, kidney, spleen, and heart; rinse in saline; blot in filter paper; weigh; and analyze for radioactivity in a gamma-counter.
4. Use radiotracing data to calculate the following parameters of conjugates behavior in vivo (for more information, *see* **refs. *3***, ***8***, and ***9***):
   a. Percent of injected dose (%ID) characterizes total uptake in a given organ and thus it shows biodistribution and effectiveness of the immunoconjugate targeting. However, this parameter does not take into account organ sizes; thus, uptake in the liver (approx 10 g in a rat) might appear far greater than the uptake in smaller organs (e.g., lung, ~1 g).
   b. To evaluate tissue selectivity of the uptake (and compare the data obtained in different animal species, as well as organs with different sizes), calculate %ID per gram (%ID/g).
   c. The ratio between %ID/g in an organ of interest and that in blood gives the localization ratio (LR) that compensates for a difference in the blood level of circulating conjugates and allows comparison of targeting between different carriers, which may have different rates of blood clearance.
   d. By dividing the LR of a specific antibody conjugate in an organ by that of the control IgG counterpart, calculate the immunospecificity index (ISI = LR_{MAb}/LR_{IgG}), the ratio between the tissue uptake of immune and nonimmune counterparts normalized to their blood level. ISI is the most objective parameter of the targeting specificity.

3.3. Results

3.3.1. Characterization of Immunoconjugates in Cell Culture

3.3.1.1. Analysis of Binding and Fate of Radiolabeled Conjugates

Site-specific binding and degradation of the conjugates by cells was determined by measuring ^{125}I in fractions of glycine elution, TCA pellet, and supernatant of cell lysates obtained from HUVECs incubated with anti-PECAM/^{125}I-catalase and IgG/^{125}I-catalase conjugates as described in **Subheading 3.1.1.** The sum of the recovered ^{125}I shows total amount of catalase associated with cells and reveals the specificity of binding of anti-PECAM conjugates, using as negative control nonspecific IgG conjugates (**Fig. 1A**). A relatively minor fraction of ^{125}I was found in the supernatant after TCA precipitation of cell lysates, indicating that catalase undergoes very modest degradation within 1 h of incubation at 37°C in endothelial cells (**Fig. 1B**).

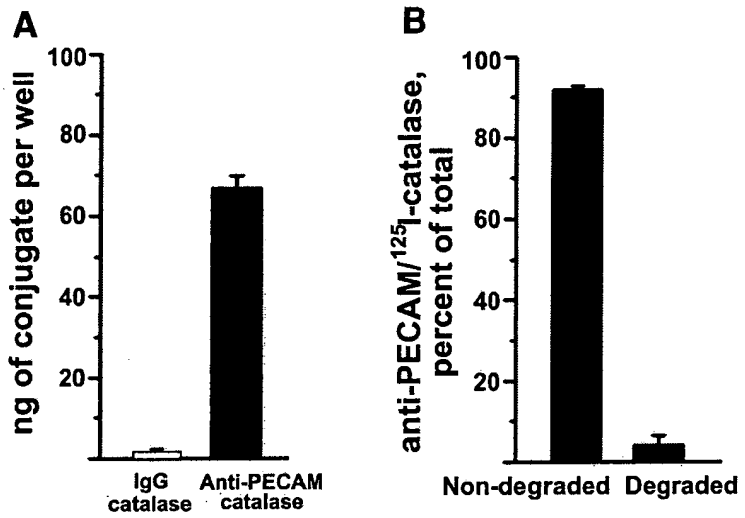

Fig. 1. Quantitative analysis of binding and degradation of radiolabeled anti-PECAM/catalase conjugates in HUVECs. HUVECs were incubated for 90 min at 37°C with anti-PECAM/^{125}I-catalase or control IgG counterpart conjugates, washed, and lysed to determine the TCA-soluble fraction of cell-bound radioactivity. The absolute amount of conjugate in the different fractions is calculated based on its specific activity as described in **Subheading 3.1.1.**

3.3.1.2. Imaging of Binding, Internalization, and Fate of Immunoconjugates by Immunofluorescence

Figure 2 shows that anti-PECAM/catalase but not IgG/catalase conjugates bind to HUVECs at 4°C, thus confirming the data obtained with ^{125}I tracing (*see* **Fig. 1**). Comparison of fluorescence and phase-contrast images indicates that anti-PECAM/catalase conjugates are located in the cell periphery, consistent with the predominant expression of PECAM-1 to the cell borders.

Moreover, in cells incubated for 1 h at 37°C with anti-PECAM/catalase conjugates, only a fraction of the conjugate was labeled before permeabilization by Texas Red-labeled secondary antibody, whereas FITC-labeled secondary antibody applied after permeabilization reveals abundant immunostaining (**Fig. 3A**). Single FITC-labeled (green) internalized conjugates are localized in the perinuclear region of the cell, whereas noninternalized double-labeled (yellow) conjugates tend to localize to the cell periphery. Semiquantitative analysis of double-labeled and single-labeled images shows that endothelial cells internalize 50% of cell-bound anti-PECAM/catalase conjugates.

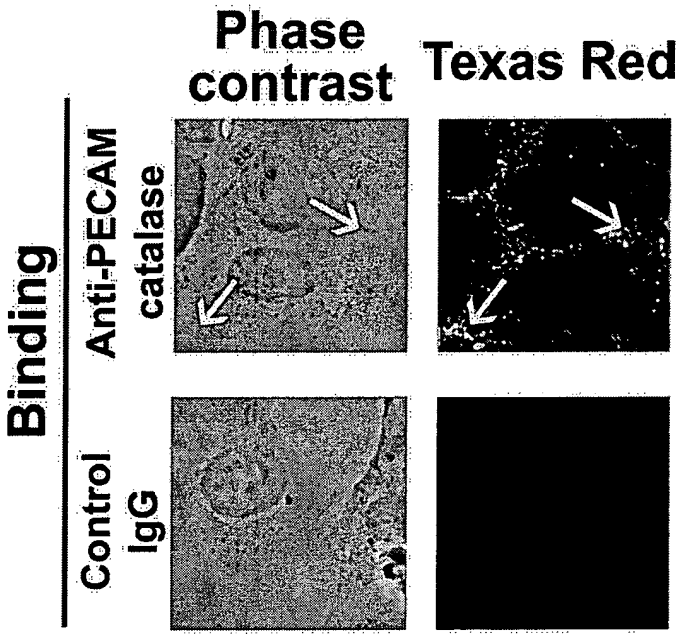

Fig. 2. Fluorescent detection of binding of anti-PECAM/catalase conjugates to HUVECs. HUVECs were incubated for 30 min at 4°C with anti-PECAM/catalase or nonspecific IgG conjugates, washed, fixed, and surface-bound anti-PECAM was stained with Texas Red goat anti-mouse IgG. The samples were analyzed by phase contrast and fluorescence microscopy. The arrows show conjugates bound to the cell surface.

To visualize and estimate degradation of internalized cargoes by fluorescence microscopy, one can retreat to use fluorescently-labeled conjugates, for example, based on FITC-labeled synthetic nanobeads used as carriers for both targeting antibodies and enzyme cargoes *(4,5)*. The advantage of this carrier is that it permits direct tracing of the conjugates in cellular compartments, including lysosomes. FITC-labeled regular immunoconjugates can also be used for this purpose, (e.g., conjugates containing FITC–streptavidin; *see* **Note 4**). A pulse-chase incubation (initial incubation 30 min at 4°C followed by removal of nonbound conjugates and incubation at 37°C), permits one to separate phases of binding, internalization, and intracellular trafficking. After internalization and fixation, surface-bound particles are counterstained using goat anti-mouse IgG conjugated to Alexa Fluor 350, followed by cell permeabilization and incubation with Texas Red-labeled goat anti-mouse IgG. This staining method

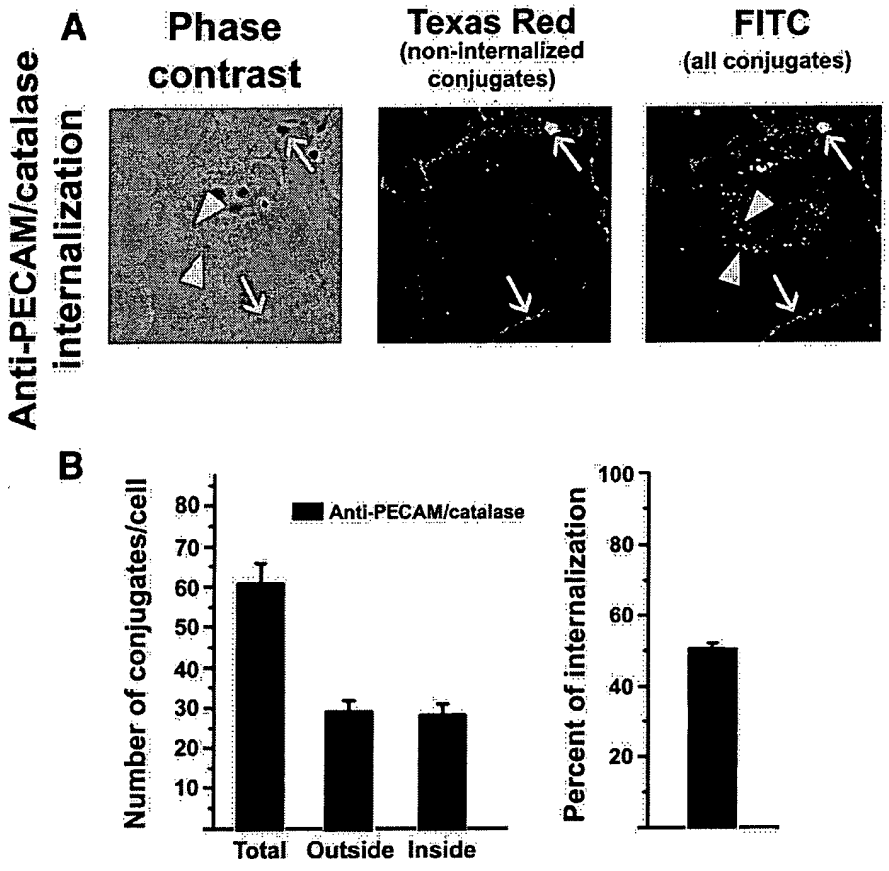

Fig. 3. Fluorescence microscopy of the uptake of anti-PECAM/catalase conjugates by HUVECs. HUVECs were incubated for 1 h at 37°C in the presence of anti-PECAM/catalase conjugates, washed, fixed, and noninternalized conjugates were stained with Texas Red-labeled goat anti-mouse IgG, followed by cell permeabilization and staining with FITC-labeled goat anti-mouse IgG. **A,** The arrows show double-labeled conjugates on the cell surface. The arrowheads show single FITC-labeled conjugates, internalized within the cell. **B,** Quantification of the experiment described above, expressed as mean and standard error (n = 10 fields, from two independent experiments).

(**Fig. 4**) distinguishes surface-bound (triple-stained, white), as well as internalized nondegraded (double-stained, yellow) and degraded conjugates (single-stained, green). The results of the particular experiment shown in **Fig. 4** indicate that conjugates are stable within the cell for 1–2 h and degrade 3 h after internalization.

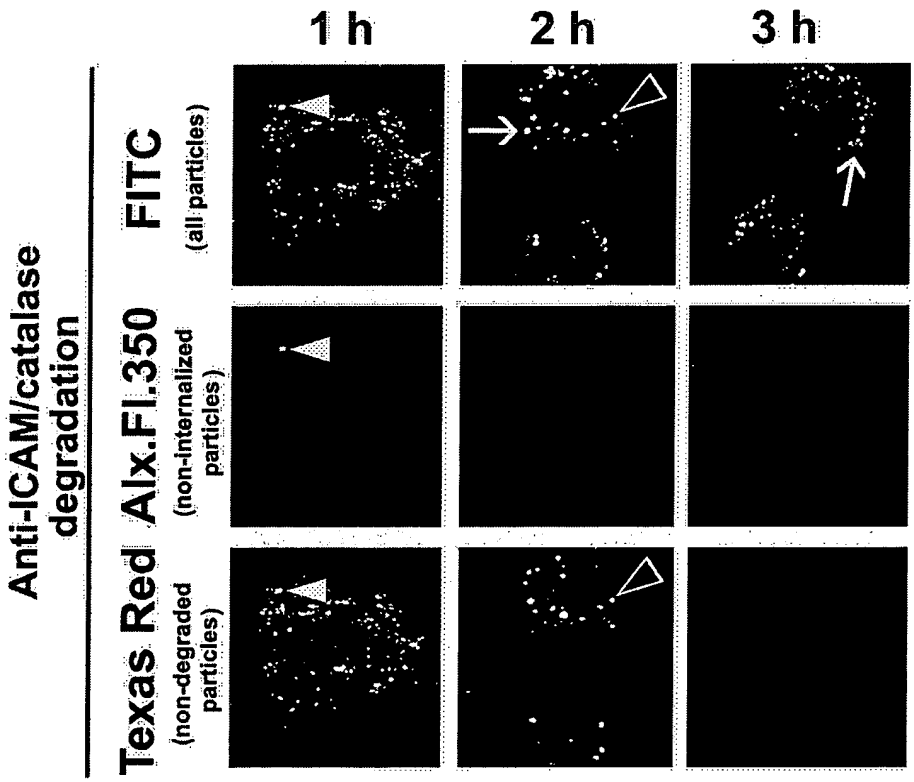

Fig. 4. Imaging of the stability of anti-ICAM/catalase nanoparticles internalized in HUVECs. HUVECs were incubated for 30 min at 4°C in the presence of FITC-labeled anti-ICAM/catalase nanoparticles to permit binding of these to the surface antigen. Then, nonbound particles were washed and the cells were incubated either for 1 h, 2 h, or 3 h at 37°C, to permit internalization and intracellular trafficking of the anti-ICAM/catalase particles. After cell fixation, noninternalized particles were stained with Alexa Fluor 350 goat anti-mouse IgG. Thereafter, the cells were permeabilized and incubated with Texas Red goat anti-mouse IgG. The samples were analyzed by fluorescence microscopy. Closed arrowheads show a triple FITC+Alexa Fluor 350+Texas Red-labeled particle, located to the cell surface. Open arrowheads show a double FITC+Texas Red-labeled particle, which indicates that the targeting antibody was not degraded after internalization within the cell. The arrow shows single FITC-labeled particles, indicating that the targeting antibody in the internalized particles has been degraded.

3.3.2. Characterization of Immunoconjugates In Vivo

3.3.2.1. Biodistribution and Pulmonary Targeting of tPA Conjugated With Anti-ICAM

Experiments with tPA conjugated with an ICAM-1 monoclonal antibody illustrate analysis of vascular immunotargeting in vivo. **Figure 5A** shows comparison of biodistribution of radiolabeled anti-ICAM/^{125}I-tPA conjugate and its components, either ^{125}I-anti-ICAM or ^{125}I-tPA, 1 h after intravenous injection in rats. Anti-ICAM and anti-ICAM/tPA conjugate display preferential uptake in the pulmonary vasculature and significant uptake in hepatic and splenic vasculature. These highly vascularized organs (especially lungs that possess about 30% of endothelial surface in the body) represent privileged targets in agreement with the fact that ICAM is constitutively expressed on the endothelial surface ***(10)***. Nonconjugated tPA shows no pulmonary targeting; in fact, its extremely rapid clearance (its half-life in rats is around 1–5 min; **ref. *11***) leads to disappearance of the tracer from blood and major organs within 1 h after injection.

3.3.2.2. Comparison of Biodistribution Attained Using Different Injection Routes

High levels of pulmonary uptake of conjugates directed against pan-endothelial determinants, such as ICAM-1, might be to the result of several reasons: (1) an extremely extended endothelial surface in the alveolar capillaries; (2) the fact that lung receives 100% of the heart blood output; or (3) the phenomenon of first-pass blood after intravenous injection. **Figure 5B** shows that injection of anti-ICAM/tPA conjugate via the left ventricle, which obviates the first pass in the lungs, produces less effective pulmonary targeting, suggesting that indeed first-pass phenomenon contributes to the pulmonary targeting. However, a high level of pulmonary uptake after left ventricle administration confirms the specificity of anti-ICAM conjugates targeting in vivo.

3.3.2.3. Evaluation of the Targeting Specificity of Immunoconjugates

Figure 6 illustrates the analysis of immunoconjugates biodistribution and targeting in rats 1 h after intravenous injection. A comparison of %ID/g in organs reveals that anti-ICAM/tPA conjugate but not IgG/tPA counterpart accumulates in the pulmonary vasculature. However, the blood level of anti-ICAM/tPA is lower than that of the nonimmune IgG/tPA counterpart, likely because of depletion of circulating blood pool by endothelial binding. The LR that compensates for differences in blood level reveals very high selectivity of anti-ICAM/tPA uptake in highly vascularized organs including liver (LR close to 3), spleen (LR exceeds 7), and especially lungs (LR close to 30). Calculation

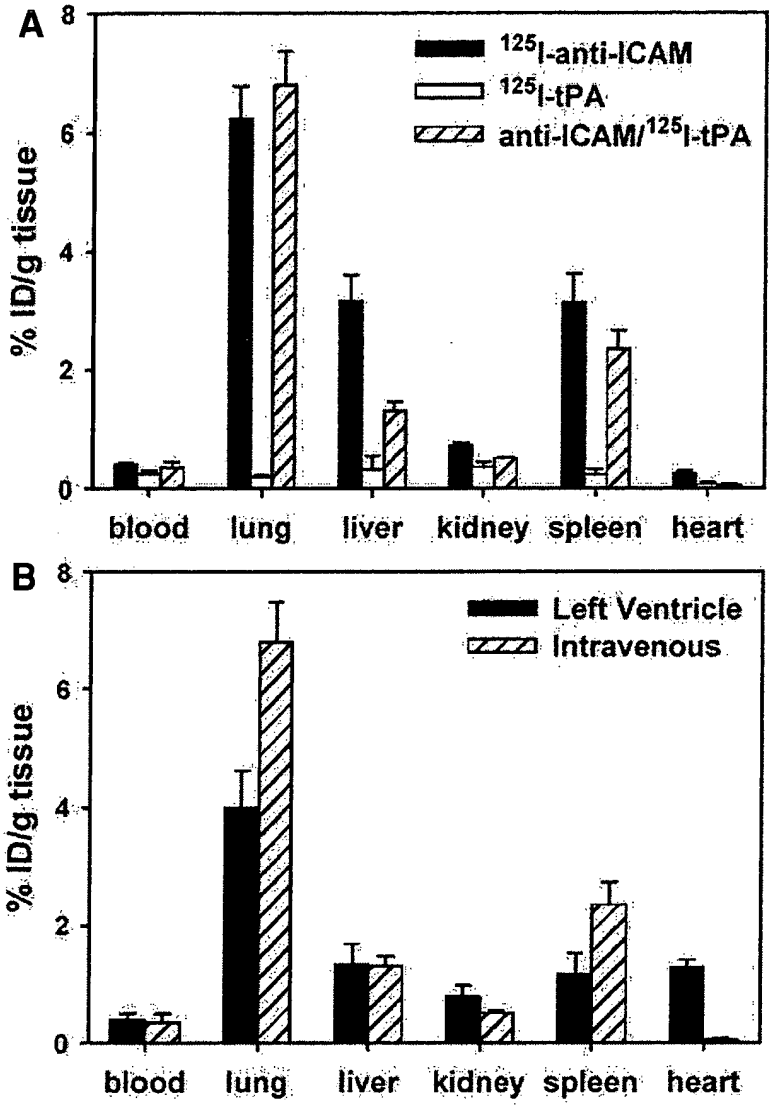

Fig. 5. Biodistribution of immunoconjugates and free components in vivo. Tracer amounts of radiolabeled proteins (approx 1 μg of radioactive material per sample) were injected intravascularly in anesthetized rats. After 1 h, animals were sacrificed and blood and organs extracted and analyzed for radioactivity. **A,** ^{125}I-anti-ICAM (black bars) or anti-ICAM/125I-tPA (hatched bars), but not free ^{125}I-tPA (white bars) accumulate in the lung, liver, and spleen after intravenous injection. **B,** Comparison of biodistribution of anti-ICAM/^{125}I-tPA after injections via the tail vein (hatched bars) or the left ventricle (black bars). Data are presented as mean ± SD, $n = 4$–9 animals per determination.

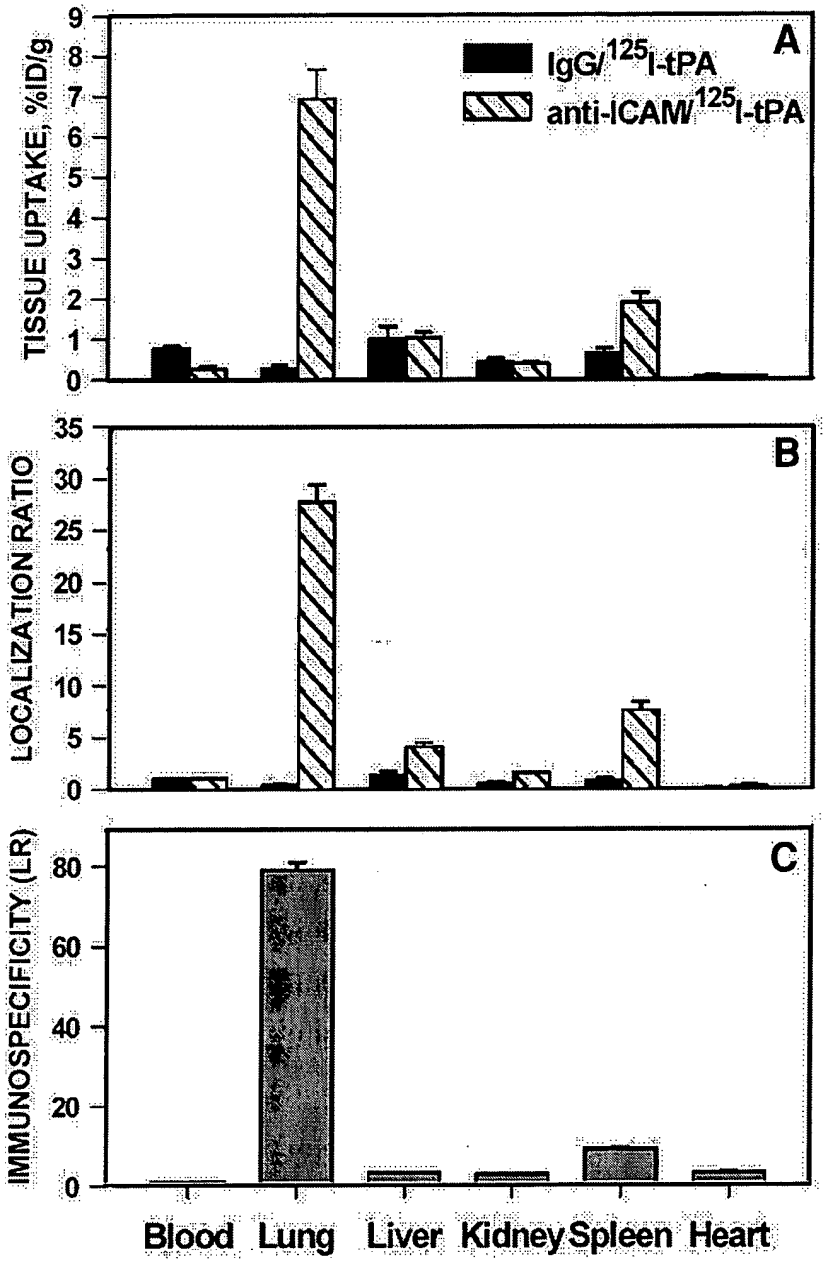

Fig. 6. Analysis of anti-ICAM/tPA biodistribution in vivo. Radioactivity in organs was analyzed 1 h after intravenous injection anti-ICAM/^{125}I-tPA (hatched bars) or control nonspecific IgG/^{125}I-tPA (black bars). The data (mean ± SD, n = 4–9) is presented as: (A) % ID/g of tissue; (B) LR; and (C) ISI. Adapted from **ref. 3**.

of ISI reveals that anti-ICAM/tPA accumulates in the lungs almost 100 times higher than IgG/tPA counterpart, thus confirming high specificity of targeting.

4. Notes

1. Uptake and trafficking of immunoconjugates within the target cells can be studied by tracing the antibody carrier, the enzymatic cargo, or both moieties. The protocols described in **Subheadings 3.1.2.1., 3.1.2.2.**, and **3.1.2.3.** trace antibody moieties using secondary antibodies against murine IgG. The same protocols can be used to trace enzyme cargo, for example, using an antibody to catalase. Moreover, conjugates directly labeled with a fluorescent probe, such as the ones based on fluorescent-labeled nanobeads or streptavidin crosslinker, are optimal because they can be visualized without additional staining. There are some specific factors that may require adjustment and optimization of the described protocols to be applied to particular conjugates and target cells of interest. Some general considerations are given below.
2. Many cell types do not adhere well to glass surfaces. Coating coverslips with a proadhesive protein (i.e., fibronectin, vitronectin, collagen) before cell seeding helps to solve this problem. A 1-h incubation with 1% gelatin solution in PBS followed by a 1-h incubation to dry coverslips up is a generic choice. The density of seeding of each cell type must be adjusted to reach confluence within the first 48 h after seeding to avoid repeated division cycles that can lead to detachment. For example, optimal density for HUVEC is 7×10^4 cell per 24 wells when seeded 48 h before the experiment. Moreover, cells tend to detach from any substrate at 4°C; thus, cold incubation should be minimal to permit binding of the conjugates. To avoid excessive detachment, pour washing medium gently and slowly on the well wall rather than directly on the cells. Glycine elution of membrane-bound conjugates may also provoke cell detachment and incubation time must be minimal (do not exceed 15 min). Inspect cell morphology and monolayer integrity by phase contrast microscopy and terminate "high-risk" exposures at the first signs of cell retraction, rounding or detachment.
3. Fixation of cells with 2% paraformaldehyde solution (10–15 min) is generally used when preparing samples for immunofluorescence, but the concentration must be optimized and can be lowed to 0.5 % to 1% if necessary to avoid disruption of the plasma membrane and partial cell permeabilization. In addition, the concentrations and incubation times of labeled antibodies given above are arbitrary and should be adjusted for particular preparations. To block nonspecific binding of labeled antibodies, preincubate fixed cells with a solution 10–20% serum of a corresponding animal species before immunostaining. To reduce nonspecific binding of the immunoconjugates (e.g., to control cells that do not express a target antigen), use incubation media containing 2–4% BSA.
4. Adjust settings for acquisition and processing of fluorescence images to optimize visualization. For instance, in the case that fluorescent signal was low, rational increase of the exposure time or brightness postacquisition can be performed, although preserving the specificity of the signal and the legitimacy of the image.

This approach helps to colocalize fluorescent signals obtained from different objects when labeled with fluorescent probes at different intensity, such as staining of a highly fluorescent FITC-labeled conjugate using secondary antibody that is relatively poorly labeled with Texas Red. Merging the images taken under similar acquisition parameters will show FITC signal masking Texas Red on the same object, not permitting visualization of a real double-labeled object and, therefore, leading to misinterpretation of the result. In addition, the choice of the fluorescent probes to reveal colocalizing objects should be made such that colors resulting from merged images permit an easy interpretation of the results. For instance, colocalization of green and red results in yellow and the three colors can be readily interpreted. However, colocalization of green and blue results in a light, bluish shade, not clearly distinguishable from the two parental colors.

5. Finally, the data on internalization and degradation of the radiolabeled conjugates should be analyzed and interpreted cautiously. For instance, multimeric conjugates can bind to cells with such high avidity that resulting large antibody/antigen clusters are difficult to disrupt by glycine elution, providing false-positive internalization result. Visualization of the uptake using double-fluorescence based techniques permits to circumvent this artifact.

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A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1

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Summary

Antibody conjugates directed against intercellular adhesion molecule (ICAM-1) or platelet-endothelial cell adhesion molecule (PECAM-1) have formed the basis for drug delivery vehicles that are specifically recognized and internalized by endothelial cells. There is increasing evidence that ICAM-1 and PECAM-1 may also play a role in cell scavenger functions and pathogen entry. To define the mechanisms that regulate ICAM-1 and PECAM-1 internalization, we examined the uptake of anti-PECAM-1 and anti-ICAM-1 conjugates by endothelial cells. We found that the conjugates must be multimeric, because monomeric anti-ICAM-1 and anti-PECAM-1 are not internalized. Newly internalized anti-ICAM-1 and anti-PECAM-1 conjugates did not colocalize with either clathrin or caveolin, and immunoconjugate internalization was not reduced by inhibitors of clathrin-mediated or caveolar endocytosis, suggesting that this is a novel endocytic pathway. Amiloride and protein kinase C (PKC) inhibitors, agents known to inhibit macropinocytosis, reduced the internalization of clustered ICAM-1 and PECAM-1. However, expression of dominant-negative dynamin-2 constructs inhibited uptake of clustered ICAM-1. Binding of anti-ICAM-1 conjugates stimulated the formation of actin stress fibers by human umbilical vein endothelial cells (HUVEC). Latrunculin, radicicol and Y27632 also inhibited internalization of clustered ICAM-1, suggesting that actin rearrangements requiring Src kinase and Rho kinase (ROCK) were required for internalization. Interestingly, these kinases are part of the signal transduction pathways that are activated when circulating leukocytes engage endothelial cell adhesion molecules, suggesting the possibility that CAM-mediated endocytosis is regulated using comparable signaling pathways.

Key words: HUVEC, Vascular endothelium, Cell adhesion, Macropinocytosis, Endocytosis

Introduction

Endothelial cells internalize natural ligands and artificial macromolecular ligands, that have been designed as carriers for specific drug and gene delivery (Danilov et al., 2001; Jacobson et al., 1996; McIntosh et al., 2002; Muzykantov et al., 1996; Spragg et al., 1997). We have previously shown that platelet-endothelial cell adhesion molecule (PECAM-1), an immunoglobulin superfamily cell adhesion molecule, can serve as a receptor for delivery of active enzymes and genetic materials to endothelial cells (Muzykantov et al., 1999; Scherpereel et al., 2001; Wiewrodt et al., 2002). Importantly, monomeric anti-PECAM-1 immunoglobulin G (IgG) and multivalent conjugates larger than 500 nm are not efficiently internalized; multivalent anti-PECAM-1 conjugates 100-300 nm in diameter are readily internalized, although molecular mechanisms that regulate the internalization of anti-PECAM-1 are not well understood (Wiewrodt et al., 2002).

It is becoming apparent that both PECAM-1 and ICAM-1 may serve as plasma membrane receptors to mediate internalization of natural ligands by different types of cells. For instance, coxsackieviruses and rhinoviruses bind ICAM-1 and are internalized (Shafren et al., 1997a), although other coreceptors may be involved in this process (Shafren et al., 1997b). HIV is internalized into brain endothelial cells by a pathway that is analogous to macropinocytosis into endocytic vesicles that also contain ICAM-1 (Liu et al., 2002). A pathway related to macropinocytosis has also been implicated in the clearance of apoptotic cell fragments by epithelial cells (Fiorentini et al., 2001). The notion that endothelial cells might help scavenge apoptotic cells is underscored by the observation that PECAM-1 is required for the binding of malaria-infected red blood cells to human umbilical vein endothelial cells (HUVEC) in culture (Treutiger et al., 1997), although these particles were too large to be endocytosed. In a recent study, PECAM-1 expressed by macrophages was found to play an important role in cellular recognition and uptake: apoptotic cells binding to macrophage PECAM-1 were efficiently phagocytosed, whereas live cells activated a signaling cascade through macrophage PECAM-1 to weaken their engagement to macrophages and enable their release (Brown et al., 2002). Cells, apoptotic fragments and viruses binding to ICAM-1 and PECAM-1 are multivalent, complex and, in the case of live cells, active partipicants in cell–cell interactions, making it difficult to discern roles for specific plasma membrane proteins as potential receptors. By contrast, anti-ICAM-1 and anti-PECAM-1 conjugates, although multivalent, will primarily engage only the cell adhesion molecule of interest, which makes them useful probes for

examining specific internalization pathways mediated by ICAM-1 or PECAM-1.

There are multiple pathways for ligand internalization involving vesicles 100-300 nm in diameter, including clathrin-mediated endocytosis and the clathrin-independent caveolae-mediated pathway (Mukherjee et al., 1997; Nichols and Lippincott-Schwartz, 2001). Each of these endocytic mechanisms differs in sensitivity to pharmacological agents, which enables the mechanism of ligand internalization to be determined. Caveolae-mediated endocytosis is a particularly important pathway in endothelial cells, where ligands such as albumin (Minshall et al., 2000) and orosomucoid (Predescu et al., 1998) are internalized via receptors clustered into caveolae and subsequently transcytosed across the endothelial barrier (McIntosh et al., 2002). There are also clathrin-independent pathways distinct from caveolar endocytosis, which mediate uptake of glycosylphosphatidylinositol (GPI)-anchored proteins, such as the folate receptor (Mayor et al., 1998) and diptheria toxin receptor (Skretting et al., 1999).

The regulation of ICAM-1 and PECAM-1 internalization by endothelial cells is not well understood at present. In particular, whether ICAM-1 and PECAM-1 are internalized by similar pathways is not known. In this study, we defined some key elements regulating the internalization of anti-ICAM-1 or anti-PECAM-1 conjugates by endothelial cells. In each case, clustering of the CAM was required for efficient internalization. Given that anti-ICAM-1 and anti-PECAM-1 conjugates did not colocalize with known endocytic coat proteins and from the analysis of the signaling pathways that regulate the uptake of anti-ICAM-1 and anti-PECAM-1 conjugates, our data suggests that that endothelial cells internalize clustered ICAM-1 and PECAM-1 using a novel endocytic pathway.

Materials and Methods

Reagents

Murine monoclonal antibodies to human ICAM-1 (R6.5) and PECAM-1 (mAb 62) were provided by Robert Rothlein (Boehringer-Ingelheim, Ridgefield, CN) and M. Nakada (Centocor, Malvern, PA), respectively. Control murine IgG was from Calbiochem (San Diego, CA). Anti-caveolin-1, anti-clathrin, anti-cholera toxin B and anti-transferrin were from Calbiochem (La Jolla, CA). Polyclonal rabbit anti-6-His-Tag was a gift from MBL (Nagoya, Japan). Secondary fluorescent antibodies were from Jackson ImmunoResearch (West Grove, PA) and Molecular Probes (Eugene, OR). Fluorescent transferrin and cholera toxin B were from Molecular Probes. Polystyrene-latex beads 100 nm in diameter, and loaded with a fluorochrome compatible with FITC fluorescence (Fluoresbrite YG microspheres), were purchased from Polysciences (Warrington, PA). Unless otherwise stated, all other reagents were from Sigma (St Louis, MO).

Cell culture

Pooled human umbilical vein endothelial cells (HUVEC) from Clonetics (San Diego, CA) were maintained in M199 medium (GibcoBRL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 15 μg/ml endothelial cell growth supplement (ECGS), 100 μg /ml heparin, 100 U/ml penicillin and 100 μg/ml streptomycin. EAhy926 cells from an endothelial-like hybrid cell line generated from HUVEC and A549 cells (Edgell et al., 1983) were cultured in DMEM medium (GibcoBRL) supplemented with 10% FBS, glutamine and antibiotics. Cultures were maintained at 37°C, 5% CO_2 and 95% relative humidity in 1% gelatin-coated tissue culture plastic. HUVEC were used between passage 4 and 5. When seeded for experiments, the cells were cultured onto 12 mm^2 gelatin-coated coverslips in 24-well plates in the absence of antibiotics and then treated with tumor necrosis factor-α (TNF-α) for at least 16 hours.

Preparation of immunobeads and immunoconjugates

Fluorescent microspheres were coated with either anti-ICAM-1, anti-PECAM-1 or control murine IgG by incubation at room temperature (RT) for 1 hour as previously described (Wiewrodt et al., 2002). The coated microspheres (immunobeads) were centrifuged to remove unbound antibodies, then resuspended in 1% bovine serum albumin-PBS and microsonicated for 20 seconds at low power. The effective immunobead diameter was determined by dynamic light scattering (DLS) using BI-90Plus particle size analyzer with BI-9000AT Digital auto-correlator (Brookhaven Instruments, Brookhaven, NY) as previously described (Wiewrodt et al., 2002). This immunobead protocol yielded uniform preparations with particle diameters ranging from 180 to 250 nm. For anti-ICAM-1 immunoconjugates, anti-ICAM-1 was biotinylated and complexed to streptavidin (90% unlabeled, 10% rhodamine labeled) in a manner equivalent to anti-PECAM-1 immunoconjugates as previously described (Wiewrodt et al., 2002). The ratio of biotinylated-ICAM-1 to streptavidin was varied to generate immunoconjugates either smaller than 500 nm or larger than 1000 nm, as determined by DLS.

Binding and uptake of anti-ICAM-1 and anti-PECAM-1 immunobeads

Confluent HUVEC or EAhy926 cells were pre-incubated overnight with 250 units of TNF-α. By flow cytometry, TNF-α treatment increased ICAM-1 expression by HUVEC and EAhy926 cells ~10-fold and had little effect on PECAM-1 expression, which is consistent with previously published results (Delisser and Albeda, 1998). The cells were then washed in serum-free medium and incubated in 1% BSA-medium containing a 1:10 dilution of either uncoated microspheres, or immunobeads coated with control murine IgG, anti-ICAM-1 or anti-PECAM-1. The cells were incubated with immunobead preparations for different time periods at 4°C or 37°C, washed in medium and fixed with 2% paraformaldehyde at RT. To distinguish between surface-bound or internalized immunobeads, nonpermeabilized fixed cells were counterstained for 30 minutes at RT with Texas Red (TxR)-conjugated goat anti-mouse IgG to produce double-labeled, yellow particles. The cells were washed in PBS, mounted onto slides with Mowiol and analyzed by fluorescence microscopy. Alexa Fluor 594-labeled cholera toxin B (FL-cholera toxin) counterstained with goat anti-cholera toxin + fluorescein rabbit anti-goat IgG was used as a control for caveolae-mediated uptake. TxR-labeled transferrin counterstained with goat anti-transferrin + fluorescein rabbit anti-goat IgG was used as a control for clathrin-mediated endocytosis.

To identify compartments containing internalized immunobeads, HUVEC monolayers were incubated with immunobeads for 1 hour at 4°C to allow surface binding, washed, then incubated at 37°C for different time periods to permit endocytosis. The cells were fixed, permeabilized and incubated with rabbit polyclonal anti-human caveolin-1, followed by incubation with goat anti-rabbit IgG conjugated to Alexa Fluor 350. Colocalization with clathrin heavy chain was done in a comparable manner, using TRITC-conjugates anti-clathrin.

For microscopy, samples mounted onto glass slides were observed using an Olympus IX70 inverted fluorescence microscope, 40× or 60× PlanApo objectives and filters optimized for fluorescent immunobeads (excitation BP460-490 nm, dichroic DM505 nm, emission BA515-

550 nm), TxR fluorescence (excitation BP530-550 nm, dichroic DM570 nm, emission BA590-800+ nm) and Alexa Fluor 450 (excitation BP360-370 nm, dichroicDM400 nm, emission BA420-460 nm) (Chroma Technology, Brattleboro, VT). Separate images for each fluorescence channel were acquired using a Hamamatsu Orca-1 CCD camera. The images were then merged and analyzed with ImagePro 3.0 imaging software (Media Cybernetics, Silver Spring, MD) as previously described (Wiewrodt et al., 2002). For quantitation, merged images of cells labeled with immunobeads were scored automatically for total green fluorescent particles and noninternalized immunobeads (double-labeled yellow particles). Uptake was calculated as the percentage of internalized immunobeads with respect to the total number of cell-associated immunobeads. Statistical significance was determined by Student's *t* test.

Mechanisms of ICAM-1- and PECAM-1-mediated uptake

Mammalian pcDNA3 expression vectors encoding for 6-His-tagged versions of human dynamin-2 [wild-type and dominant-negative forms (K44A), (PH*)] were gifts from Drs S. Schmid (Scripps Research Institute, La Jolla, CA) (Altschuler et al., 1998) and M. Lemmon (U Penn School of Medicine, Philadelphia, PA) (Lee et al., 1999). EAhy926 endothelial cells were transfected using Lipofectin (GibcoBRL) complexed to 1.5 μg DNA/dish encoding either dynamin-2, dynamin-2(K44A) or dynamin-2(PH*). Each construct includes a 6-His amino terminus tag to distinguish it from endogenous dynamin-2. Twelve hours after transfection, the cells were stimulated with TNF-α, incubated for 36 hours and then anti-ICAM-1 or anti-PECAM-1 uptake was determined by double labeling as described above. Following labeling of surface-bound material, the cells were permeabilized with 0.2% Triton X-100 and then immunostained using 5 μg/ml rabbit anti-6-His-Tag and goat anti-rabbit IgG conjugated to Alexa Fluor 350 to identify cells expressing recombinant dynamin-2.

For studies using pharmacological inhibitors, TNF-α-stimulated HUVEC or EAhy926 were pre-incubated for 30 minutes at 37°C in the presence of one of the following agents: 50 μM monodansylcadaverine (MDC), 1 μg/ml filipin, 50 μM genistein, 3 mM amiloride, 25 μM monensin, 0.5 mM cytochalasin D, 0.1 μM latrunculin A, 20 μM nocodazole, 5 μM bisindolyl-maleimide-1 (BIM-1), 10 μM 1-(5-isoquiniline sulphonyl)-2-methylpiperazine (H7), 0.1 μM phorbol 12-myristate 13-acetate (PMA), 10 μM radicicol, 10 μM Y-27346 or 0.5 μM wortmannin (Barreiro et al., 2002; Fujimoto et al., 2000; Parton et al., 1994; Racoosin and Swanson, 1989; Sahai and Marshall, 2002; Schlegel et al., 1982; Schnitzer et al., 1994; Swanson, 1989; Torgersen et al., 2001; Watanabe et al., 2001; West et al., 1989). Molecular targets for selected inhibitors are shown in Fig. 10. The concentration of each agent was selected using literature values and was optimized qualitatively by fluorescence microscopy (not shown). Also, we examined the effectiveness of each agent using suitable controls (e.g. Fig. 4). Potassium depletion was done by pre-incubating the cells for 15 minutes in potassium depletion buffer (0.14 M NaCl, 2 mM $CaCl_2$, 1 mg/ml glucose, 20 mM HEPES, pH 7.4) diluted 1:1 with water to make it hypotonic (Koval et al., 1998). After treatment, the cells were incubated with immunobeads, cholera toxin or transferrin at 37°C, in the presence of K^+-depletion buffer or the given inhibitors, and then fixed and double labeled for surface-bound material as described above.

Results

Monomeric antibodies to PECAM-1 are poorly internalized by endothelial cells (Muzykantov et al., 1999; Wiewrodt et al., 2002). Multivalent anti-PECAM-1 conjugates with a diameter of 100-300 nm are readily internalized, but the efficiency of internalization decreases with increasing conjugate size (Wiewrodt et al., 2002). To test whether this is the case for anti-ICAM-1 conjugates, we examined the internalization of anti-ICAM-1 conjugates in two different size ranges by TNF-α-stimulated HUVEC (Fig. 1A,B). Consistent with our previous results using anti-PECAM-1 conjugates, we found that anti-ICAM-1 conjugates with a diameter less than 500 nm were

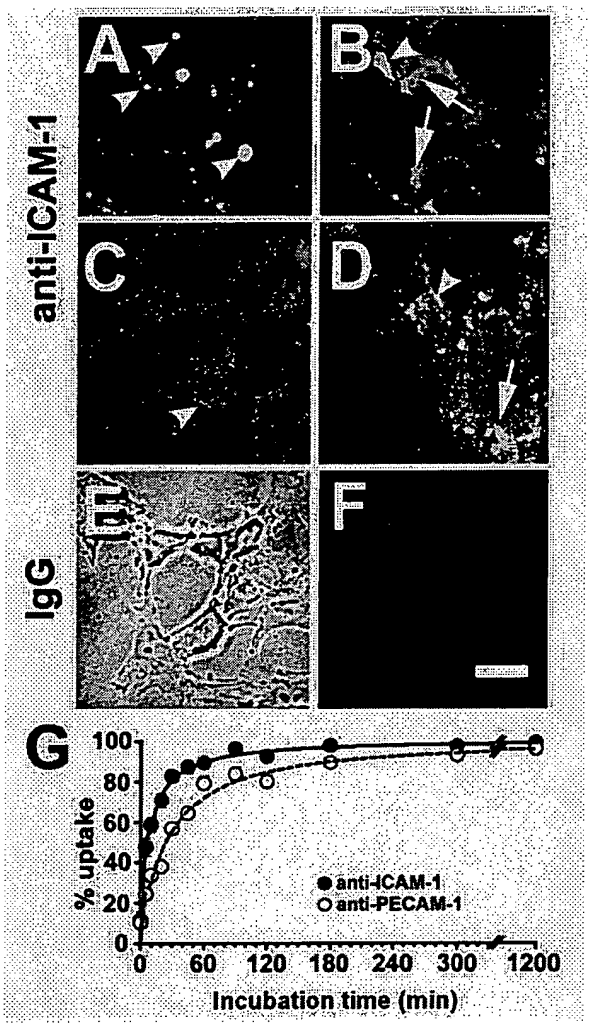

Fig. 1. Small ICAM-1 immunoconjugates are internalized by HUVEC. HUVEC were treated with 250 U TNF-α for 24 hours. Confluent monolayers were incubated at either 4°C (c) or 37°C (A,C-F) in the presence of either large (A; >1000 nm diameter), small (b; <500 nm diameter) biotin-anti-ICAM-1/streptavidin conjugates, anti-ICAM-1 immunobeads (C,D) or beads previously coated with control murine IgG (E,F). The cells were subsequently washed, fixed and counterstained with fluorescent goat anti-mouse IgG. Merged images corresponding to representative samples were pseudocolored to show single-labeled, internalized immunoconjugates/immunobeads as green (arrows) and double-labeled immunoconjugates/immunobeads on the cell surface as yellow (arrowheads). The phase-contrast image shown in e corresponds to the fluorescence image shown in F. Bar, 10 μm. (G) Uptake of anti-ICAM-1 (●) and anti-PECAM-1 (○) immunobeads by TNF-α-activated HUVEC was determined for different incubation times as the mean percentage of internalized (single labeled) immunobeads per cell. Error bars corresponding to s.d. were smaller than the size of the symbols used for the graph.

internalized by HUVEC, whereas conjugates with diameter greater than 1000 nm showed little, if any, internalization. Because antibody conjugates show a broad distribution of particle sizes (Wiewrodt et al., 2002), we performed subsequent experiments using anti-ICAM-1 and anti-PECAM-1 immunobeads, which have a more uniform diameter in the size range that allows internalization (Wiewrodt et al., 2002). Anti-ICAM-1 immunobeads were internalized by HUVEC when incubated at 37 °C but not at 4°C (Fig. 1C,D). Similar results were obtained using anti-PECAM-1 beads (S.M., R.W. and A.T. et al., unpublished). Following internalization, there was further clustering of conjugates and immunobeads, which was probably due to endosome fusion events that occurred as the particles were transported along the endocytic pathway.

On average, ~125 immunobeads/cell were internalized after a 1 hour incubation at 37°C. Fig. 1G shows that uptake of anti-ICAM-1 immunobeads was more rapid (~10 minutes half time for uptake) than that of anti-PECAM-1 immunobeads (~20 minutes half time for uptake). Similar results were obtained for anti-ICAM-1 and anti-PECAM-1 immunobeads internalized by naïve HUVEC, indicating that TNF-α had little effect on the mechanism of internalization. This also suggests that the mechanism for internalization is not sensitive to the surface density of ICAM-1, as virtually all of the anti-ICAM-1 immunobeads were internalized after 30 minutes at 37°C, despite the difference in binding in the absence (42±15 beads/cell) or presence of TNF-α (165±54 beads/cell). Also, as found for anti-PECAM-1, monomeric anti-ICAM-1 was not internalized (S.M., R.W. and A.T. et al., unpublished).

Because dynamin-2 is frequently involved in vesicle-mediated internalization and phagocytosis (Altschuler et al., 1998; Gold et al., 1999; Henley et al., 1998; Lee et al., 1999), we examined the role for dynamin-2 in uptake of anti-ICAM-1 immunobeads. Given the low transfection rate of HUVEC, we used the endothelial-like cell line, EAhy926, which showed a 40% transfection efficiency using Lipofectin. EAhy926 cells internalized anti-ICAM-1 immunobeads in a manner comparable to HUVEC (Fig. 2). These cells were transiently transfected to express amino-terminal 6-His-tagged forms of either wild-type or dominant-negative dynamin-2 (K44A or PH*). Expression of recombinant proteins was identified by immunofluorescence, using an antibody that recognizes the 6-His epitope (Fig. 2). Cells transfected with wild-type dynamin-2 showed no effect on the uptake of anti-ICAM-1 immunobeads compared with control cells. By contrast, cells expressing ether dynamin-2(K44A) or dynamin-2(PH*) showed less anti-ICAM-1 immunobead uptake than control cells, suggesting that uptake of these immunobeads required dynamin-2 (Fig. 2). This was not due to a net decrease in immunobead binding, which was equivalent for nontransfected EAhy926 cells (23±3 beads/cell) and EAhy926 cells expressing wild-type (24±16 beads/cell) or mutant dynamin-2 [25±4 beads/cell (PH*), 14±5 beads/cell (K44A)].

We also found that the dominant-negative dynamin-2 constructs inhibited the uptake of Alexa Fluor 594-conjugated cholera toxin (FL-cholera toxin, S.M., R.W. and A.T. et al., unpublished) that is internalized by caveolae-mediated endocytosis (Schnitzer et al., 1994). However, few, if any, anti-ICAM-1 immunobeads colocalized with caveolin-1-positive structures, regardless of whether the immunobeads were bound to the plasma membrane or internalized by HUVEC (Fig. 3).

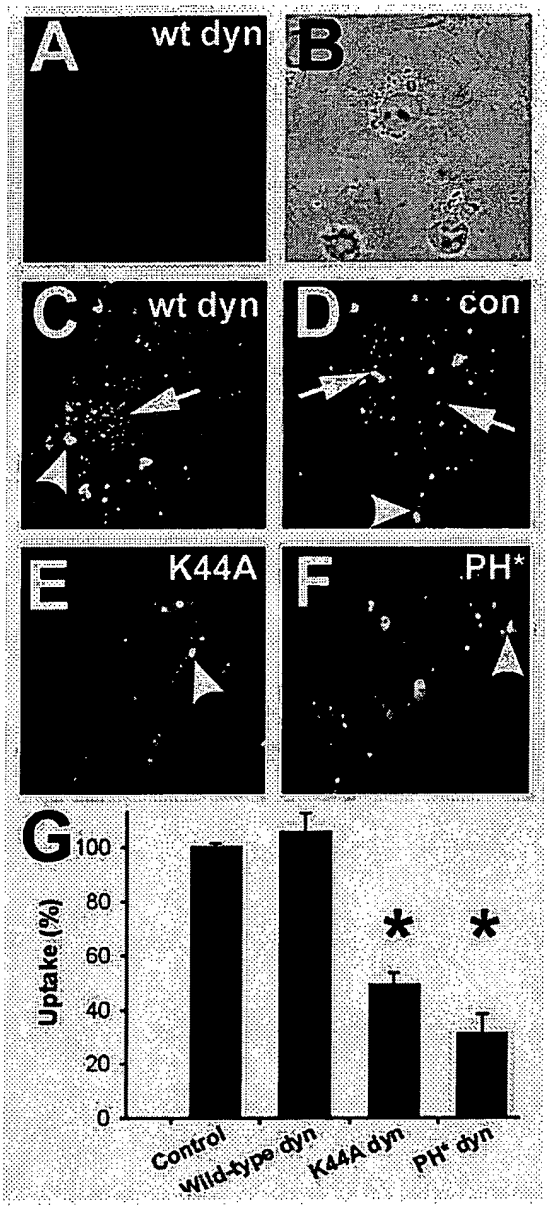

Fig. 2. Anti-ICAM-1 immunobead uptake is inhibited by dominant-negative dynamin constructs. EAhy926 cells were transfected with 1.5 μg of DNA encoding either wild-type (A-C) or dominant-negative (K44A; e, PH*; f) dynamin-2. Nontransfected cells are shown in (D). Twelve hours post-transfection, cells were stimulated with TNF-α for 36 hours and then incubated for 2 hours at 37°C with anti-ICAM-1 immunobeads. The cells were then washed, fixed and surface-bound particles were counterstained with TxR goat anti-mouse IgG. The cells were then permeabilized and stained with rabbit anti-6-His antibody followed by Alexa Fluor 350 goat anti-rabbit IgG to identify transfected cells expressing dynamin (A). The corresponding phase-contrast image is shown in (B). Merged images corresponding to representative samples of transfected (C,E,F) or control (D) cells are shown, where single-labeled, internalized immunobeads are green (arrows) and double-labeled immunobeads on the cell surface are yellow (arrowheads). Blue fluorescence of transfected cells is omitted in panels (C,E,F) to enable better visualization of red and green fluorescence. (G) The percentage of immunobead uptake was calculated as described as mean±s.d. *$P<0.05$.

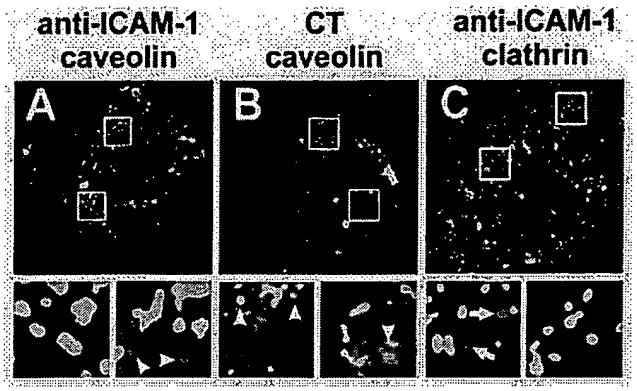

Fig. 3. Anti-ICAM-1 immunobeads do not colocalize with caveolin or clathrin. TNF-α-stimulated HUVEC were incubated with control anti-ICAM-1 immunobeads (A,C) or Alexa 594-conjugated cholera toxin B subunit (B) for 15 minutes at 37°C. The cells were then washed and fixed, and surface-bound material was counterstained with TxR goat anti-mouse IgG (A,C) or goat anti-cholera toxin followed by fluorescein rabbit anti-goat IgG (B). After permeabilization, the cells were then labeled with rabbit anti-human caveolin-followed by Alexa 350-conjugated goat anti-rabbit IgG (A,B) or TRITC-conjugated anti-clathrin heavy chain (C). Insets show images magnified twofold. The image color channels were selected to facilitate the comparison between panels in the figure, and they are: green, internalized immunobeads or cholera toxin; blue, surface-bound immunobeads or cholera toxin; red, caveolin-1 (arrowheads) or clathrin (arrows). There was little, if any, colocalization of anti-ICAM-1 immunobeads with caveolin-1 or clathrin, as evidenced by the lack of yellow labeling in A and C and areas showing internalized immunobeads with little caveolin-1 or clathrin nearby (see insets).

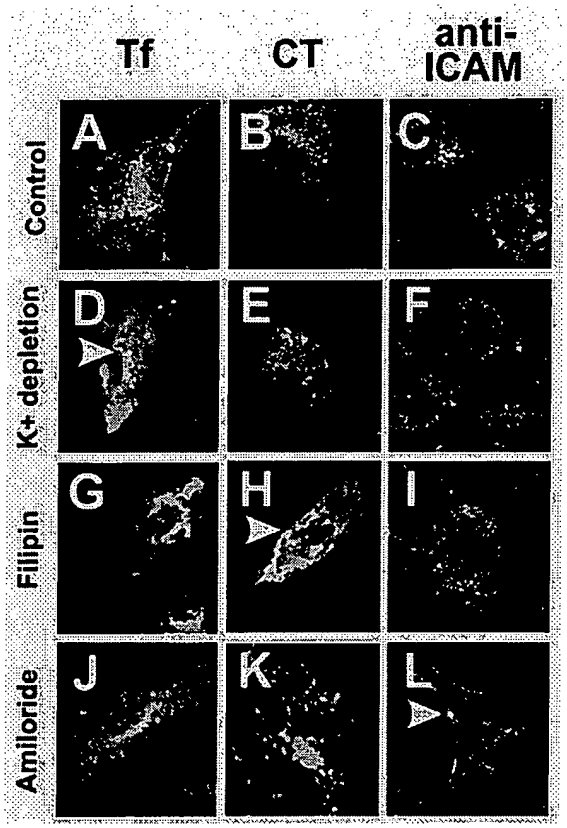

Fig. 4. Anti-ICAM-1 conjugates are not internalized by clathrin or caveolae-mediated endocytosis. TNF-α-activated HUVEC were untreated (A-C), potassium-depleted (D-F), or treated for 30 minutes at 37°C with 1 μg/ml filipin (G-I), or 3 mM amiloride (J-L). The cells were incubated in the presence or absence of inhibitors for 1 hour at 37°C with fluorescent transferrin (Tf: A,D,G,J), fluorescent cholera toxin (CT: B,E,H,K) or anti-ICAM-1 immunobeads (C,F,I,L), then fixed and counterstained to double-label surface-bound material (yellow, arrowheads). As shown, potassium depletion specifically inhibited transferrin uptake by clathrin-mediated endocytosis (D), filipin specifically inhibited caveolar uptake of cholera toxin (H) and amiloride specifically inhibited uptake of anti-ICAM-1 immunobeads (L).

By contrast, FL-cholera toxin showed extensive colocalization with caveolin, which is consistent with previously published reports (Puri et al., 2001). Newly internalized anti-ICAM-1 immunobeads also did not colocalize with clathrin. In fact, there was a low level of clathrin immunofluorescence shown by HUVEC, consistent with a less-dominant role for clathrin-coated pits in endocytosis than caveolae-mediated pathways in endothelial cells (Schubert et al., 2001). Thus, despite being a dynamin-dependent process, anti-ICAM-1 conjugate uptake by endothelial cells was unlikely to be through caveolae- or clathrin-coated vesicles.

We therefore used a series of pharmacological inhibitors to further characterize internalization of anti-ICAM-1 and anti-PECAM-1 conjugates by HUVEC. The specificity of different inhibitors was confirmed using fluorescent transferrin and cholera toxin as controls for clathrin-mediated and caveolar endocytosis, respectively (Fig. 4). As shown in Fig. 5, inhibitors of clathrin-mediated transferrin endocytosis (MDC, potassium depletion) did not inhibit the uptake of anti-ICAM-1 or anti-PECAM-1 immunobeads. Furthermore, inhibitors of caveolae-dependent cholera toxin uptake (filipin, genestein) were not effective at inhibiting anti-ICAM-1 or anti-PECAM-1 immunobead internalization. Because uptake of anti-ICAM-1 and anti-PECAM-1 immunobeads appeared to be through a unique internalization pathway, we examined the effect of other inhibitors on immunobead endocytosis. Previous work has indicated that amiloride, an inhibitor of the sodium/proton pump, can inhibit macropinocytosis by dendritic cells (West et al., 1989). Amiloride had little effect on internalization of FL-cholera toxin or transferrin, suggesting that it did not inhibit caveolae- or clathrin-mediated endocytosis (Fig. 4). However, amiloride inhibited uptake of anti-ICAM-1 and anti-PECAM-1 immunobeads by HUVEC (55±15% and 60±9%, respectively) and by EAhy926 cells (34±9% and 24±3% inhibition, respectively. Anti-ICAM-1 immunobead binding was equivalent for control (125±21 beads/cell) and amiloride-treated HUVEC (154±37 beads/cell), suggesting that amiloride did not decrease ICAM-1 surface expression. TNF-α stimulation was not required, given that amiloride inhibited anti-PECAM-1 immunobead uptake by naïve HUVEC by 50.5±6.2%. Also, anti-ICAM-1 immunobead uptake by amiloride-treated HUVEC remained inhibited during a 3 hour (43.8±2.9%) and 5 hour incubation (50.6±5.2%), suggesting that amiloride altered the extent of immunobead uptake, rather than uptake kinetics. Furthermore, this was not likely to be due to an effect on ion homeostasis, given that the ionophore

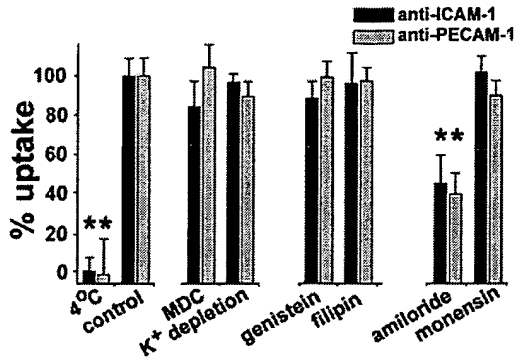

Fig. 5. Effect of endocytosis inhibitors on anti-ICAM-1 and anti-PECAM-1 uptake. Uptake of anti-ICAM-1 and anti-PECAM-1 immunobeads was quantified as mean±s.d. by fluorescence microscopy using control cells, potassium-depleted cells or cells pretreated for 30 minutes at 37°C before incubation with immunobeads with either 50 μM MDC, 50 μM genistein, 1 μg/ml filipin, 3 mM amiloride or 25 μM monensin. Cells incubated with anti-ICAM-1 or anti-PECAM-1 immunobeads at 4°C are controls for no internalization. *P<0.05.

monensin had little, if any, effect on anti-ICAM-1 and anti-PECAM-1 immunobead uptake (Fig. 5).

Because protein kinase C (PKC) has been reported to play a pivotal role in macropinocytosis and phagocytosis by macrophages (Araki et al., 1996; Larsen et al., 2000; Swanson, 1989), we tested the effect of PKC inhibitors on immunobead internalization by HUVEC. As shown in Fig. 6, the PKC inhibitors BIM-1 and H-7 inhibited immunobead uptake by ~30% and ~60%, respectively. H-7 treatment also inhibited the uptake of anti-ICAM-1 and anti-PECAM-1 by EAhy926 cells by 55±11% and 48±7%, respectively. HUVEC pretreated with 0.1 μM PMA for 30 minutes (conditions that stimulate PKC activity) showed a high level of anti-ICAM-1 and anti-PECAM-1 internalization (>95%), and the total level of immunobead uptake by HUVEC was stimulated nearly twofold. Anti-ICAM-1 immunobead binding was equivalent for control (125±21 beads/cell) and BIM-1-treated HUVEC (140±7 beads/cell), suggesting that BIM-1 did not decrease ICAM-1 surface expression. Stimulation by TNF-α was not required, since inhibiting PK-C activity also inhibited uptake of anti-PECAM-1 immunobeads by naïve HUVEC (47.5±9.8%) and uptake of anti-PECAM-1 immunobeads by naïve HUVEC was enhanced 1.3-fold by PMA. Also, anti-ICAM-1 immunobead uptake by BIM-1-treated HUVEC remained inhibited during a 3 hour (39.6±4.2%) and 5 hour incubation (42.9±5.2%), suggesting that BIM-1 altered the extent of immunobead uptake, rather than uptake kinetics. Taken together, these results are consistent with internalization of anti-ICAM-1 and anti-PECAM-1 immunobeads by a PKC-dependent pathway. However, since H-7 may also interfere with actin-based contractility (Volberg et al., 1994), this effect may also contribute to the inhibition of uptake.

In fact, the formation of F actin stress fibers is frequently associated with ICAM-1 crosslinking (Thompson et al., 2002; Wang and Doerschuk, 2002). Therefore, we examined the effect of anti-ICAM-1 immunobeads on the formation of actin stress fibers by HUVEC. As shown in Fig. 7, stress fibers were rapidly

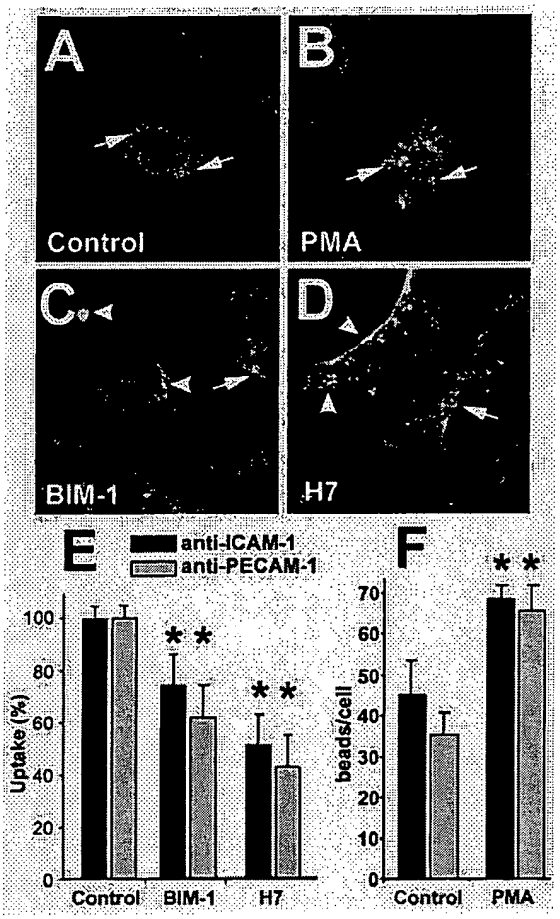

Fig. 6. Uptake of anti-ICAM-1 or anti-PECAM-1 immunobeads is PKC-mediated. TNF-α-activated HUVEC were treated for 30 minutes with vehicle alone (A), 0.1 μM PMA (B), 0.1 μM BIM-1 (C) or 10 μM H-7 (D), then incubated with anti-ICAM-1 or anti-PECAM-1 immunobeads for 1 hour at 37°C, then fixed and immunostained to double-label surface-bound material (yellow, arrowheads). Arrows denote internalized immunobeads. Uptake of anti-ICAM-1 and anti-PECAM-1 immunobeads was quantified as mean±s.d. by fluorescence microscopy for these treatments, expressed as a percentage of immunobead uptake for the PKC inhibitors (E). For PMA, this is expressed as the total number of internalized particles per cell (F), as the percent internalization was equivalent for control and PMA stimulated cells. *P<0.05.

induced by anti-ICAM-1 immunobead binding. Immunobeads appeared to align along actin stress fibers before internalization; this is shown most prominently by the blue labeled immunobeads in Fig. 7C,D. Vesicles containing internalized immunobeads continued to be associated with stress fibers after internalization and remained associated with actin during a 3 hour incubation. Few, if any, anti-ICAM-1 immunobeads induced formation of an actin coat (phagocytic cup) at the site of internalization, which is a hallmark of phagocytosis and macropinocytosis (Grimmer et al., 2002; Lee and Knecht, 2002). Note that this is probably not a problem with the detection of actin coats, as a previous study found that ~20% of 0.2 μm beads internalized by Fc receptors in macrophages were associated with actin coats (Koval et al., 1998).

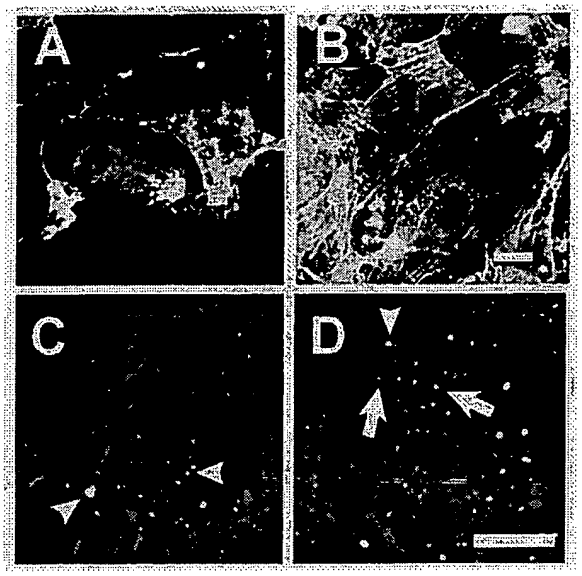

Fig. 7. Internalized anti-ICAM-1 immunobeads associate with the actin cytoskeleton. (A,b) HUVEC incubated in either the absence (A) or presence (B) of anti-ICAM-1 immunobeads for 15 minutes were fixed and then treated with rhodamine phalloidin to label filamentous actin. Note the stimulation of actin stress fibers by anti-ICAM-1 immunobeads. Bar, 10 μm. (C,D) HUVEC were incubated with anti-ICAM-1 immunobeads for 15 (C) or 30 (D) minutes, fixed, then immunostained to double-label surface-bound material (blue, arrowheads). Arrows denote internalized immunobeads in vesicles associated with stress fibers. Bar, 10 μm.

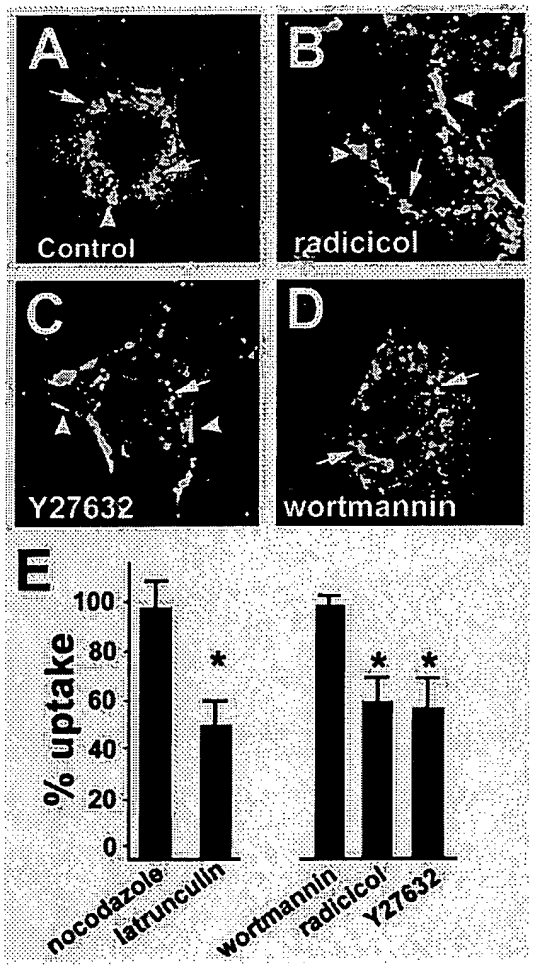

Fig. 8. Uptake of anti-ICAM-1 immunobeads requires actin regulatory proteins. TNF-α activated HUVEC were treated for 30 minutes with vehicle alone (A), 10 μM latrunculin A, 20 μM nocodazole, 10 μM radicicol (B), 10 μM Y-27632 (C) or 0.5 μM wortmannin (D), then incubated with anti-ICAM-1 immunobeads for 1 hour at 37°C, then fixed and immunostained to double-label surface-bound material (yellow, arrowheads). Arrows denote internalized immunobeads. Uptake of anti-ICAM-1 immunobeads was quantified as mean±s.d. by fluorescence microscopy for these treatments, expressed as a percentage of immunobead uptake. Uptake required both Src kinase activity and ROCK activity, since it was inhibited by radicicol and Y27632, but did not appear to require PI-3 kinase activity, as wortmannin had no measurable effect on uptake. *$P<0.05$.

Given the dramatic association of anti-ICAM-1 immunobeads with actin in HUVEC, we also examined the cytoskeletal requirements for the uptake of anti-ICAM-1 and anti-PECAM-1 immunobeads. In contrast to macropinocytosis by macrophages (Racoosin and Swanson, 1992), microtubules were not required for the internalization of anti-ICAM-1 immunobeads by HUVEC, since internalization was not inhibited by nocodazole (Fig. 8). Also, nocodazole did not significantly inhibit the uptake of anti-PECAM-1 immunobeads (12±9% inhibition). Cytochalasin D, which caps short actin filaments, had little effect on the uptake of anti-ICAM-1 immunobeads (18±7% inhibition) or anti-PECAM-1 immunobeads (8±8% inhibition). However, the more effective actin depolymerizing agent, latrunculin, inhibited anti-ICAM-1 immunobead uptake (Fig. 8) and uptake of anti-PECAM-1 immunobeads (69±15% inhibition).

We also examined inhibitors that affect kinases known to play a role in regulating actin organization. In contrast to macropinocytosis by phagocytes (Araki et al., 1996; West et al., 2000), wortmanin had little, if any, effect on uptake of anti-ICAM-1 immunobeads by HUVEC, suggesting that PI3-kinases were not involved in immunobead uptake. However, as shown in Fig. 8, uptake of anti-ICAM-1 immunobeads was inhibited by the Src kinase inhibitor radicicol and the ROCK inhibitor Y27632, consistent with the notion that both the PKC pathway and Rho pathway regulate actin cytoskeletal rearrangements required for internalizaton of clustered ICAM-1. Four inhibitors of anti-ICAM-1 immunobead internalization – latrunculin, amiloride, radicicol and Y27632 – all inhibit the formation of actin stress fibers induced by anti-ICAM-1 immunobead binding (Fig. 9), underscoring the correlation of uptake by HUVEC with actin mobilization.

Discussion

In this study, we found that endothelial cells internalize clustered ICAM-1 and clustered PECAM-1 by a novel endocytic pathway (Table 1). CAM-mediated endocytosis was distinct from caveolae-mediated uptake (McIntosh et al., 2002; Minshall et al., 2000; Predescu et al., 1998) as it was not inhibited by genestein or filipin and the bound conjugates did

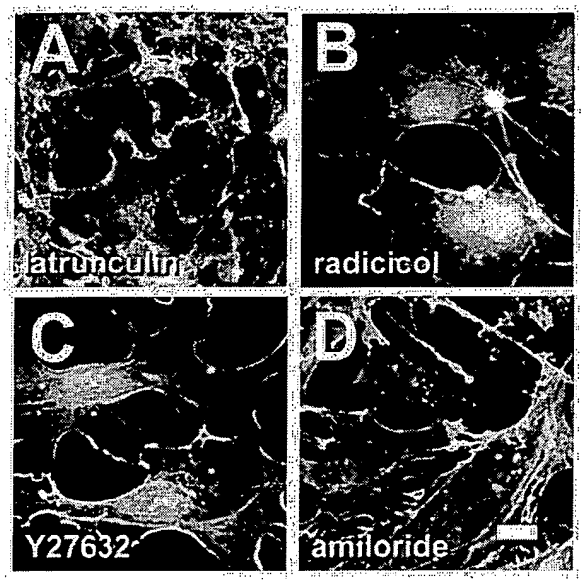

Fig. 9. Agents that inhibit anti-ICAM-1 immunobead uptake disrupt actin rearrangements induced by immunobeads. HUVEC were pretreated with 10 μM latrunculin A (A), 10 μM radicicol (B), 10 μM Y-27632 (C) or 3 mM amiloride (D), incubated with anti-ICAM-1 immunobeads for 15 minutes then fixed and stained for filamentous actin using rhodamine phalloidin. Each of these agents that inhibit uptake of anti-ICAM-1 immunobeads also inhibited actin stress-fiber formation. Bar, 10 μm.

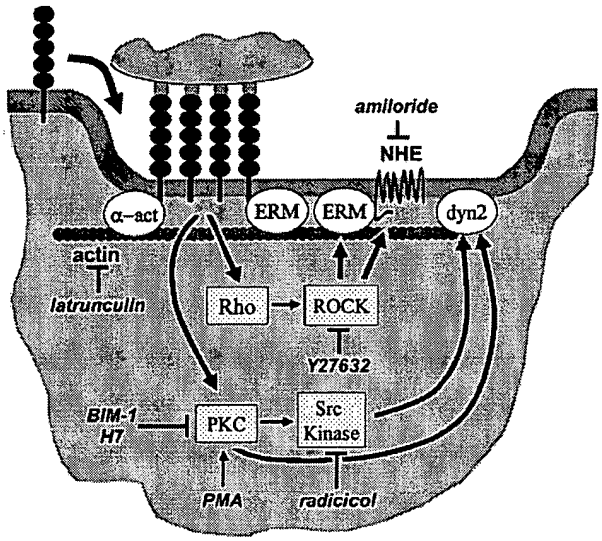

Fig. 10. Model for uptake mediated by cell adhesion molecules. The model shown is for signaling pathways stimulated when monomeric ICAM-1 or anti-PECAM-1 are clustered by binding to immunoconjugates. Pharmacological inhibitors and activators are indicated by italics. Cell adhesion molecules have the capacity to bind proteins that mediate direct interactions with the actin cytoskeleton, such as α-actinin (α-act) and ERM proteins. On the basis of our inhibitor data and results from the literature, we propose that clustering of ICAM-1 or PECAM-1 can also stimulate PKC, Src kinase and ROCK signal transduction pathways. This could help regulate the recruitment of other cofactors, such as dynamin-2 (dyn2), to the plasma membrane. Also, ERM proteins and NHE transporters are downstream targets for phosphorylation by the Rho/ROCK pathway, which might further serve to recruit actin to sites of immunoconjugate uptake in response to ICAM-1 or PECAM-1 clustering.

not colocalize with caveolin. Also, uptake of anti-ICAM-1 and anti-PECAM-1 conjugates was distinct from clathrin-mediated endocytosis, because it was not inhibited by potassium depletion or MDC and the conjugates did not colocalize with clathrin (Hansen et al., 1993; Schlegel et al., 1982). Whether CAM-mediated endocytosis is specific for endothelial cells remains to be determined, although PECAM-1 transfected REN mesothelioma cells show a similar endocytic pathway (Wiewrodt et al., 2002).

On the basis of amiloride sensitivity and PKC dependence, internalization of clustered anti-ICAM-1 and anti-PECAM-1 seemed to be related to macropinocytosis (Lamaze and Schmid, 1995; Nichols and Lippincott-Schwartz, 2001; Orth et al., 2002; Swanson and Watts, 1995), a pathway that is not typically associated with endothelial cells. Nonetheless, CAM-mediated endocytosis was distinct from 'classical' macropinocytosis, on the basis of several criteria (Table 1). For example, in contrast to the dynamin-2 requirement we observed for uptake of anti-ICAM-1 immunobeads by EAhy926 endothelial cells, the K44A dominant-negative dynamin-2 did not inhibit macropinocytosis by fibroblasts (Orth et al., 2002). Although dynamin is required for endocytosis (Altschuler et al., 1998; Henley et al., 1998; Lee et al., 1999) and phagocytosis (Gold et al., 1999), CAM-mediated endocytosis differed in other ways from these processes. For instance, anti-ICAM-1 immunobeads did not colocalize with either clathrin or caveolin (Fig. 4). Also, uptake of anti-ICAM-1 immunobeads did not require PI3K activity, which is needed for phagocytosis (Araki et al., 1996; Cox et al., 1999), as well as macropinocytosis (West et al., 2000).

The uptake of anti-ICAM-1 and anti-PECAM-1 conjugates required clustering of cell adhesion molecules (Muzykantov et al., 1999; Wiewrodt et al., 2002). ICAM-1 clustering has been found to stimulate multiple intracellular signaling pathways (Adamson et al., 1999; Etienne et al., 1998), including a PKC signaling pathway that results in the phosphorylation of cytoskeletal and focal adhesion proteins, thereby enabling actin filament rearrangement (Etienne-Manneville et al., 2000). Consistent with this, anti-ICAM immunobeads induced actin stress fiber formation and were associated with stress fibers before internalization (Fig. 7). Both Src kinase and ROCK activity are required for CAM-mediated endocytosis and these inhibitors also inhibited stress fiber formation induced by anti-ICAM-1 immunobeads (Fig. 9). ROCK activity also enables remodeling of F-actin attached to adherence junctions and controls their stability (Sahai and Marshall, 2002). This also provides a potential link between ICAM-1 or PECAM-1 clustering, Src kinase, PKC and dynamin-2 (Fig. 10), as dynamins are downstream targets for Src kinase (Ahn et al., 2002) and PKC (Powell et al., 2000).

Dynamin, via interactions with endophilin and profilin, helps recruit actin to sites of endocytic activity (Farsad et al., 2001; Witke et al., 1998). Proteins in the ezrin-radixin-moesin (ERM) family are also good candidates to link internalization

Table 1. Comparison of CAM-mediated endocytosis with phagocytosis and macropinocytosis

	CR-mediated phagocytosis	FcR-mediated phagocytosis	Macropinocytosis	CAM-mediated endocytosis	Distinct from‡
Internalize particles >1 μm	Yes	Yes	Yes	No	CFM
Receptor clustering	+*	+	–	+	M
Dynamin-2	+	+	–	+	M
NHE	–	–	+	+	CF
PKC	+	+	+	+	n
Actin	+	+	+	+	n
Actin cup	+	+	+	–	CFM
Microtubules	+	–	+	–	CM
Src kinase	–	+	+	+	C
PI-3 kinase	+	+	+	–	CFM
Rho kinase	+	–	?	+	F

Table adapted from Caron and Hall (Caron and Hall, 2001) and expanded. For references to specific elements, see text.

*+, required for uptake; –, not required for uptake; ?, unknown.

‡C, distinct from complement receptor (CR)-mediated phagocytosis; F, distinct from FcR-mediated phagocytosis; M, distinct from macropinocytosis; n, none, common to all listed pathways.

of ICAM-1 and PECAM-1 to the actin cytoskeleton (Bretscher et al., 1997; Cao et al., 1999). For instance, ezrin binds directly to the C-terminus of ICAM-1 (Heiska et al., 1998). Another actin binding protein, α-actinin, has also been shown to bind to the C-terminus of ICAM-1 (Carpen et al., 1992). Perhaps signaling induced by ICAM-1 or PECAM-1 clustering can indirectly recruit ERM proteins to the plasma membrane. For instance, phosphorylation of ERM proteins by a ROCK-dependent pathway can help recruit them to the plasma membrane (Hirao et al., 1996). Intriguingly, ROCK activity has been associated with complement receptor-mediated phagocytosis, but is not required for Fc receptor-mediated endocytosis (Olazabal et al., 2002). ROCK has also been shown to phosphorylate sodium proton exchangers (NHE) to enhance binding of ERM proteins (Denker et al., 2000). Both processes might correspond to the ROCK requirement for uptake of clustered ICAM-1 or PECAM-1 (Fig. 10). This is further suggested by the ability of amiloride to inhibit uptake of clustered ICAM-1 and PECAM-1, since amiloride can disrupt the association of ERM proteins with NHE, an effect that is independent of ion channel activity (Denker et al., 2000; Putney et al., 2002).

Another major distinction from macropinocytosis and phagocytosis is that uptake of anti-ICAM-1 and anti-PECAM-1 conjugates larger than 500 nm in diameter was poor (Fig. 1) (Wiewrodt et al., 2002). Also, anti-ICAM-1 immunobeads smaller than 500 nm diameter did not induce the formation of an actin cup or coat (Fig. 7), which is typically induced by larger particles internalized by phagocytosis (Koval et al., 1998), suggesting that formation of an actin coat is crucial for internalization of larger particles. Although the mechanisms that cells use to control the size threshold for internalization is not known at present, given that ICAM-1 and PECAM-1 primarily regulate cell–cell contacts, a small size threshold for internalization may be a means by which endothelial cells avoid engulfing other cells.

Consistent with a size threshold for ICAM-1-mediated internalization, an ICAM-1 enriched structure is formed at the contact site between lymphocytes and HUVEC, where the endothelial cell appears to partially engulf the lymphocyte (Barreiro et al., 2002). Furthermore, the lymphocyte-endothelial cell docking structure requires ROCK activity, but not phosphatidylinositol 3-kinase (PI-3 kinase) (Barreiro et al., 2002), comparable to our observations for anti-ICAM-1 and anti-PECAM-1 immunobeads (Fig. 9). Whether plasma membrane internalization is part of the mechanism required to maintain this docking structure is not known at present. One possibility is that CAM-mediated endocytosis might help to remodel cell–cell junctions as leukocytes migrate along endothelial cells. If so, this might be analogous to the turnover of gap junctions, which is mediated by the engulfment of cell–cell junctions sites to create endocytic vesicles in the 200-500 nm diameter size range (Gaietta et al., 2002; Jordan et al., 2001).

The size threshold for internalization of clustered ICAM-1 and PECAM-1 might also enable endothelial cells to distinguish small apoptotic fragments from intact cells bound to the endothelial cell surface, such as other endothelial or blood cells (Barreiro et al., 2002; DeLisser and Albelda, 1998; Johnson-Leger et al., 2000; Worthylake and Burridge, 2001). The notion that endothelial cells could also scavenge apoptotic fragments via a pathway comparable to CAM-mediated endocytosis is appealing (Brown et al., 2002; Treutiger et al., 1997); however, whether this is the case remains to be determined.

Understanding the mechanisms that regulate uptake of anti-ICAM-1 and anti-PECAM-1 conjugates will probably help to extend the utility of these agents as the basis for endothelium-specific drug-targeting vehicles (Li et al., 2000; Muzykantov et al., 1999; Scherpereel et al., 2002; Scherpereel et al., 2001; Wiewrodt et al., 2002). For instance, inhibitors of conjugate uptake might help to increase their stability by reducing the extent of delivery to lysosomes and other degradative compartments. Animal studies combining the agents used in this work with the administration of pharmacologically active, enzyme-carrying anti-ICAM or anti-PECAM conjugates will be used to determine the feasibility of this approach.

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ICAM-directed vascular immunotargeting of antithrombotic agents to the endothelial luminal surface

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Drug targeting to a highly expressed, noninternalizable determinant up-regulated on the perturbed endothelium may help to manage inflammation and thrombosis. We tested whether inter-cellular adhesion molecule-1 (ICAM-1) targeting is suitable to deliver antithrombotic drugs to the pulmonary vascular lumen. ICAM-1 antibodies bind to the surface of endothelial cells in culture, in perfused lungs, and in vivo. Proinflammatory cytokines enhance anti-ICAM binding to the endothelium without inducing internalization. ^{125}I-labeled anti-ICAM and a reporter enzyme (β-Gal) conjugated to anti-ICAM bind to endothelium and accumulate in the lungs after intravenous administration in rats and mice. Anti-ICAM is seen to localize predominantly on the luminal surface of the pulmonary endothelium by electron microscopy. We studied the pharmacological effect of ICAM-directed targeting of tissue-type plasminogen activator (tPA). Anti-ICAM/tPA, but not control IgG/tPA, conjugate accumulates in the rat lungs, where it exerts plasminogen activator activity and dissolves fibrin microemboli. Therefore, ICAM may serve as a target for drug delivery to endothelium, for example, for pulmonary thromboprophylaxis. Enhanced drug delivery to sites of inflammation and the potential anti-inflammatory effect of blocking ICAM-1 may enhance the benefit of this targeting strategy. (Blood. 2003;101:3977-3984)

Introduction

Thrombosis and inflammation are often intertwined processes that contribute to cardiovascular morbidity and mortality. In many cases, the pulmonary vasculature is the major site of vascular inflammation and thrombosis, and considerable efforts have been expended to develop strategies to target drugs to this site. Yet current methods to manage inflammation-related thrombosis remain suboptimal.[1-3] For example, targeting fibrin and activated platelets promotes the delivery of antithrombotic agents to existing blood clots, for example, in coronary vessels.[4,5] However, targeting components of preformed clots has afforded only modest improvements in experimental models, likely due to limited penetration,[6] and such clot-targeting strategies are unlikely to be useful for thromboprophylaxis.

Targeted delivery of antithrombotic drugs to the vascular lumen, including those involved by inflammation, prior to clot formation may permit thromboprophylaxis in patients with a high propensity for thrombosis. Theoretically, overexpression of certain antithrombotic proteins by vascular endothelial cells themselves would help to achieve this goal.[7-9] However, gene therapies currently are not suitable to manage acute conditions.[10]

Immunotargeting of therapeutic proteins may provide a complementary strategy suitable for more immediate interventions. Antibodies to diverse determinants are being explored as affinity carriers for drug targeting to endothelium.[11-15] A poorly internalizable, high-density, and stably exposed determinant on the endothelial surface, up-regulated and functionally involved in vascular thrombosis and inflammation, would provide an ideal target for antithrombotic proteins. Previous data indirectly suggest that inter-cellular adhesion molecule-1 (ICAM) may provide such a target to deliver anti-inflammatory and, perhaps, antithrombotic agents.[16-20] However, neither the endothelial internalization of ICAM antibodies (anti-ICAM) nor the tissue distribution, localization, activity, and effects of ICAM-targeted therapeutics have been characterized.

In this work we studied ICAM-directed immunotargeting to endothelium in cell cultures, perfused lungs, and animals, and found that (1) anti-ICAM is not internalized efficiently by the endothelium, (2) cytokine up-regulation of ICAM expression augments surface targeting, but not internalization, of anti-ICAM, (3) anti-ICAM can be used to produce either internalizable (100-200 nm diameter) or noninternalizable (approximately 1 μm) conjugates, and (4) ICAM targeting delivers active tPA to the pulmonary vascular lumen and facilitates intravascular fibrinolysis.

Materials and methods

The materials used were $Na^{125}I$ and $Na^{131}I$ from Perkin-Elmer (Boston, MA), iodogen, streptavidin (SA), and 6-biotinylaminocaproic acid N-hydroxysuccinimide ester from Pierce (Rockford, IL), human recombinant tPA from Genentech (San Francisco, CA), β-galactosidase-streptavidin

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conjugate (SA-β-Gal) from Sigma (St Louis, MO), chromogenic tPA substrate, Spectrozyme tPA, a kind gift from American Diagnostica (Greenwich, CT), and fluorescein-labeled transferrin or SA from Molecular Probes (Eugene, OR). Monoclonal (IgG) antibodies were mAb R6.5,[21] mAb 1A29,[22] and mAb YN1[23] against human, rat, and murine ICAM-1; mAb 9B9 against human and rat angiotensin-converting enzyme (ACE)[24]; and mAb 1009 and mAb 311 against human and murine thrombomodulin, TM.[11] Antibodies against IgG (fluorescent-labeled or gold-conjugated) were from Jackson Immunoresearch (West Grove, PA) and Amersham (Piscataway, NJ).

Conjugation and size determination

Antibodies, IgG, and tissue-type plasminogen activator (tPA) were biotinylated and radiolabeled using Iodogen without loss of activity, as described.[25] The number of biotin residues per molecule of protein was determined by Immunopure-HABA assay (Pierce) as per manufacturer instructions. Biotinylated tPA or β-Gal was coupled to biotinylated antibodies using streptavidin cross-linking following a 3-step procedure described in detail previously.[25-27] The size of the resulting conjugates was determined by dynamic light scattering, as described.[25-27] The conjugates are designated hereafter as anti-ICAM/β-Gal and IgG/β-Gal or anti-ICAM/tPA and IgG/tPA.

Cell culture experiments

Surface binding and intracellular uptake of ^{125}I-labeled antibodies were measured in cultures of human umbilical vein endothelial cells (HUVECs, from Clonetics) and a human mesothelioma cell line expressing ICAM-1 (REN cells) as described previously for anti–platelet-endothelial cell adhesion molecule (PECAM).[28] Control and tumor necrosis factor α (TNFα)–challenged cells were incubated for 1 hour at either 4°C or 37°C with ^{125}I–anti-ICAM, ^{125}I–anti-ACE, ^{125}I–anti-TM, or ^{125}I–control IgG. After washing, surface-bound antibodies were eluted with glycine, while internalized antibodies were measured in the cell lysates.

Suspended control or TNFα-treated HUVEC and REN cells were incubated with 30 μg/mL anti-ICAM or anti-TM for 1 hour at 4°C, washed, counterstained with fluorescein isothiocyanate (FITC)–labeled goat anti–mouse IgG (30 minutes at 4°C), resuspended in phosphate-buffered saline (PBS), and analyzed by fluorescence-activated cell-sorter scanner (FACS).

Cellular localization of anti-ICAM was visualized using immunofluorescence at magnification × 60 or × 40. TNFα-treated cells were incubated with 10 μg/mL anti-ICAM for 1 hour at either 4°C or 37°C in 1% bovine serum albumin (BSA)–containing medium. After washing and fixation, the surface-associated anti-ICAM was stained with Texas Red–labeled goat anti–mouse IgG. Thereafter, internalized anti-ICAM was counterstained in permeabilized cells using FITC-labeled goat anti–mouse IgG. Fluorescein-labeled transferrin was used as a control for internalizable ligand in parallel wells. In separate experiments, rat pulmonary microvascular endothelial cells (RPMVECs) were incubated with anti-ICAM/SA–Texas Red conjugates of different sizes (100-200 nm or approximately 1 μm) for 1 hour at 37°C. After cell fixation, surface-bound conjugates were counterstained with an FITC-labeled goat anti–mouse IgG.

Experiments in isolated perfused rat lungs (IPL)

Lungs were isolated from anesthetized male 170-200 g Sprague-Dawley rats following protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) and were ventilated and perfused for 1 hour at 37°C or 4°C with Krebs ringer buffer (KRB)–BSA buffer containing ^{125}I-labeled antibodies (1 μg, unless indicated otherwise), followed by nonrecirculating perfusion with KRB-BSA, as described.[29,30] In separate experiments, 100 μg nonradiolabeled, biotinylated anti-ICAM or anti-ACE was perfused for 1 hour at 37°C. ^{125}I-labeled streptavidin (^{125}I-SA) was added to the perfusate immediately after washing the unbound antibody or after an additional 60 minutes of nonrecycling perfusion to measure surface-accessible b–anti-ICAM.

^{125}I–anti-ICAM biodistribution in rats

Anesthetized rats and mice were killed 1 hour after a tail-vein injection of a mixture of ^{125}I–anti-ICAM and ^{131}I-IgG (10 μg each), and the radioactivity in blood and major organs (washed with saline, blotted dry, and weighed) was measured to calculate the parameters of targeting: percent of injected dose per organ (%ID) or per gram (%ID/g), organ-to-blood ratio (localization ratio, LR), and immunospecificity index (ISI) (see Murciano et al[29] and Danilov et al[31] for details).

Biodistribution and tissue localization of anti-ICAM and anti-ICAM/β-Gal conjugates in animals

The tissue localization of enzymatically active anti-ICAM/β-Gal 1 hour after the tail-vein injection of 100 μg conjugate in BALB/c mice was visualized by histological analysis of X-Gal staining in the tissues, as described previously for anti-PECAM/β-Gal conjugate.[26] In a similar experiment, lungs were processed for electron microscopy and developed using a gold-conjugated secondary antibody, as described.[26]

Characterization of tPA activity in lung tissue

Anti-ICAM/tPA conjugate or control preparations (IgG/tPA and tPA) were perfused in IPL for 1 hour; unbound materials were eliminated by a 5-minute nonrecycling perfusion. In one series, lungs were perfused with 3 μg ^{125}I-labeled tPA conjugated to either anti-ICAM or IgG, and the radioactivity was measured. In the next series, aliquots of lung homogenates obtained after perfusion of 100 μg of unlabeled tPA conjugates were added to ^{125}I-labeled fibrin clots, formed as described previously,[30] and the release of ^{125}Iodine into the supernatant at 37°C was measured. In the next series, 750 μL of a 0.4 mM/L solution of a chromogenic tPA substrate was infused into the lung via the pulmonary artery for 20 minutes, and the optical density at 405 nm in the outflow perfusate was measured. In the last series, a suspension of ^{125}I-labeled fibrin microemboli (^{125}I-ME, 3-10 micron diameter), prepared as described previously,[32] was infused into the common pulmonary artery after perfusion with either anti-ICAM/tPA or IgG/tPA. ^{125}I-ME lodge and degrade slowly in intact isolated perfused rat lungs.[30] The lungs were perfused for 1 hour with buffer containing 20% plasma as a source of plasminogen, and the residual radioactivity in the lungs was measured.

Statistics

A *t* test or a one-way analysis of variance (ANOVA) (SigmaStat 2.0) was used to determine statistically significant differences ($P < .05$) between groups. Post hoc testing was performed with Fisher Least Square difference test. Data are shown as mean ± SEM unless otherwise stated.

Results

Endothelial cells internalize anti-ICAM inefficiently

^{125}I–anti-ICAM, but not control IgG, bound specifically to unstimulated endothelial cells (HUVECs) (Figure 1A). Eighty-five percent of the ^{125}I–anti-ICAM bound to cells at 37°C was eluted by glycine one hour later, compared with 90%-95% bound at 4°C (not shown).

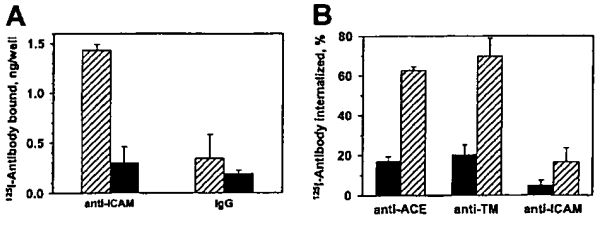

Figure 1. Resting endothelial cells bind but do not internalize ^{125}I–anti-ICAM. (A) HUVECs were incubated with ^{125}I–anti-ICAM or ^{125}I-IgG (1 hour, 37°C), and radioactivity was determined in the surface fraction (glycine elution, ▨) and in the cell lysates (■). (B) Percent of internalization of ^{125}I-labeled antibodies against ACE, TM, and ICAM-1 by HUVECs at either 4°C (■) or 37°C (▨). The data are expressed as means ± SD (n = 3).

Consistent with this, approximately 10% of bound anti-ICAM was internalized by 60 minutes at 37°C versus approximately 60% of ^{125}I–anti-ACE and ^{125}I–anti-TM (Figure 1B). Therefore, resting endothelial cells internalize anti-ICAM inefficiently, leaving approximately 90% of the antibody on the cell surface.

Anti-ICAM uptake in the perfused rat lungs (IPL)

We then asked whether anti-ICAM was handled similarly by intact vascular endothelium under flow. Rat IPLs were perfused with ^{125}I–anti-ICAM or control ^{125}I-IgG in a blood-free buffer. ^{125}I–anti-ICAM bound specifically to the lungs (Figure 2A), reaching saturation at approximately 10 μg per gram of tissue. Scatchard analysis (inset) revealed that rat lungs contain approximately 5×10^{13} anti-ICAM binding sites per gram (approximately 1.5-2.5×10^5 binding sites per endothelial cell).

We then examined the internalization of anti-ICAM and anti-ACE in IPL. Pulmonary uptake of ^{125}I–anti-ACE was markedly lower at 6°C than at 37°C, likely due to inhibition of the energy-dependent uptake of antibody. In contrast, practically the same uptake of ^{125}I–anti-ICAM was seen at 6°C and at 37°C (Figure 2B). This result reflects minor, if any, contribution of an energy-dependent internalization pathway for anti-ICAM in IPL.

The IPL setting permits sequential perfusion of ^{125}I-SA immediately or 1 hour after biotinylated antibodies, to test the accessibility of endothelium-bound antibodies to the circulation. Binding of ^{125}I-SA in the lungs was reduced by 70% when biotinylated anti-ACE was allowed to remain in the vasculature for 1 hour at 37°C, indicating antibody disappearance from the lumen. In contrast, binding of ^{125}I-SA after perfusion of biotinylated anti-ICAM did not diminish with time, indicating that the endothelium-bound anti-ICAM remains accessible from the lumen at 37°C (Figure 2C). Therefore, pulmonary endothelium under flow conditions avidly binds but internalizes anti-ICAM poorly.

TNFα stimulates anti-ICAM binding, but not internalization

TNFα augmented anti-ICAM binding to HUVECs but inhibited anti-TM binding, while REN cells, which do not express thrombomodulin, bound anti-ICAM constitutively at a relatively high level that was augmented further by TNFα (FACS analysis, Figure 3A).

FACS results were confirmed by ^{125}Iodine tracing studies using HUVEC monolayers. TNFα markedly augmented binding of ^{125}I–anti-ICAM but not control ^{125}I-IgG (Figure 3B). However, the intracellular uptake of ^{125}I–anti-ICAM by TNFα-stimulated cells after a 1-hour incubation at 37°C was equivalent in HUVEC

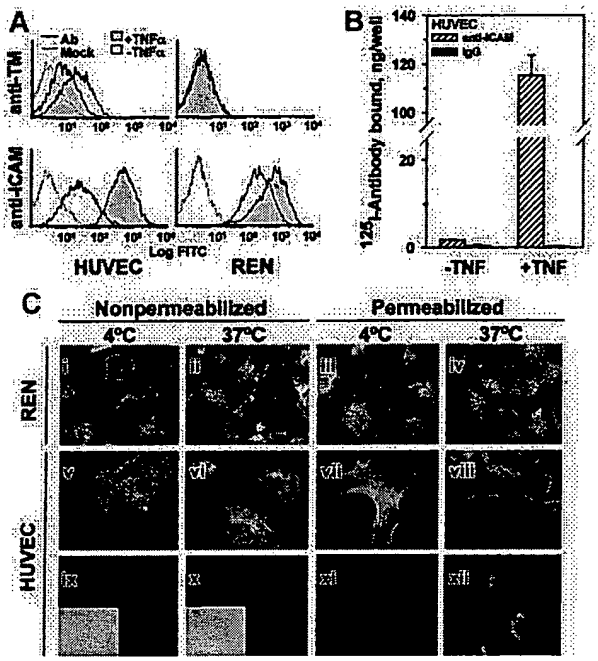

Figure 3. TNFα increases anti-ICAM binding but not internalization by endothelial and mesothelioma cells. (A) FACS analysis using anti-ICAM and anti-TM. TNFα suppresses expression of thrombomodulin (TM, upper panels) by HUVECs and stimulates that of ICAM-1 (lower panels) by HUVEC and REN cells. Dashed line: antibody-free medium; resting (open histogram) or TNFα-challenged (shaded histogram) cells. (B) ^{125}I–anti-ICAM binding to resting and TNFα-treated HUVEC monolayer. The data are shown as means ± SD, n = 4. (C) Fluorescent micrographs (× 60) of TNFα-stimulated cells incubated with anti-ICAM. The cells were incubated at 4°C or 37°C with anti-ICAM (panels i-viii), antibody-free medium (ix, x) or transferrin (xi, xii). After washing and fixation the cells were sequentially stained with Texas Red secondary antibody, permeabilized, and counterstained with FITC-labeled secondary antibody (yellow, surface-bound anti-ICAM; green, internalized anti-ICAM). On the left, the nonpermeabilized cells were stained with both Texas Red and FITC-labeled antibodies (positive control for surface staining, yellow color). Green color corresponds to the intracellular staining (see panels xi and xii showing staining of HUVECs incubated with fluorescein-labeled transferrin). Insets of subpanels ix and x show phase contrast images in controls. Original magnification, × 60; insets minimized to ⅛ of original size.

(11.6% ± 0.7%) and REN cells (10.1% ± 2.7%); background levels at 4°C were 5.1% ± 0.8% and 3.2% ± 0.6%, respectively.

These radiotracer data showing minimal (no more than 10%) internalization of anti-ICAM by cytokine-stimulated cells were confirmed by immunofluorescence microscopy. Figure 3C shows typical images of TNFα-stimulated HUVEC and REN cells

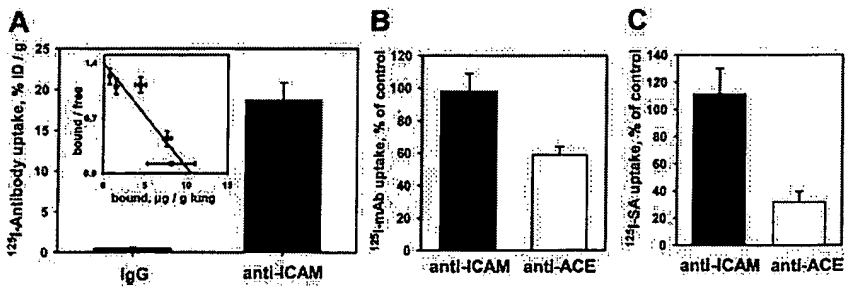

Figure 2. ^{125}I–anti-ICAM accumulates without internalization in the isolated rat lungs. (A) Accumulation of ^{125}I–anti-ICAM or ^{125}I-IgG perfused for 1 hour at 37°C. Inset shows a Scatchard analysis of ^{125}I–anti-ICAM binding. (B) Temperature dependence of anti-ICAM uptake (■) and anti-ACE uptake (□) in the lungs. At 4°C, the pulmonary uptake of ^{125}I–anti-ACE is inhibited, whereas the uptake of ^{125}I–anti-ICAM is not affected (uptake at 4°C is shown as percent of the 100% control value attained at 37°C). (C) Disappearance of anti-ICAM (■) and anti-ACE (□) from the luminal surface in the lungs perfused at 37°C. After accumulation in the lungs, biotin–anti-ICAM, but not biotin–anti-ACE, is accessible to the blood for a prolonged time. ^{125}I-streptavidin (^{125}I-SA) was perfused in the lungs either immediately after biotinylated antibody accumulation or after 60 minutes of additional perfusion at 37°C with antibody-free buffer. Data of ^{125}I-SA uptake after 60 minutes delay are shown as percent of that observed immediately after biotinylated antibody accumulation (100% level). All data are shown as means ± SEM; n = 4.

incubated with anti-ICAM for 1 hour at either 4°C or 37°C and stained before or after permeabilization with Texas Red and FITC-labeled secondary antibody. The staining of intact and permeabilized cells was essentially identical, showing predominantly dual (yellow) labeling of the surface-bound anti-ICAM both at 4°C and 37°C, with no appreciable green staining (representing internalized anti-ICAM). As a control, the intracellular staining of HUVECs incubated with internalizable fluorescein-labeled transferrin was evident at 37°C but not at 4°C (Figure 3Bxi-xii). Therefore, TNFα markedly up-regulates anti-ICAM binding to endothelial and mesothelial cells, but does not augment anti-ICAM internalization.

Biodistribution of radiolabeled anti-ICAM in vivo

^{125}I–anti-ICAM, but not ^{131}I-IgG, accumulated in the lungs after intravenous injection in rats (Figure 4) and in mice (not shown). Significant uptake also was seen in the liver and spleen, but anti-ICAM uptake per gram of tissue was always greatest in the lungs. Pulmonary uptake of anti-ICAM was 20%-30% lower after intra-arterial injection (not shown).

We analyzed the specificity of anti-ICAM targeting to pulmonary tissue. In rats, the pulmonary uptake of ^{125}I–anti-ICAM was approximately 17% ID/g (Figure 4A), an immunospecificity index ($ISI_{\%ID/g}$, ratio of %ID/g of anti-ICAM to that of IgG) of approximately 25, 10-fold higher than that in the liver and spleen (Figure 4B). The blood level of anti-ICAM was lower than that of control IgG, likely due to depletion of the circulating pool. The anti-ICAM pulmonary localization ratio (LR, tissue-to-blood ratio) was approximately 50 (Figure 4C), while the IgG LR was approximately 0.2. Therefore, the pulmonary ISI_{LR} calculated using anti-ICAM and IgG LR, thereby correcting for the blood level, was approximately 250 (Figure 4D).

Effects of proinflammatory challenges on anti-ICAM targeting

Endotoxin facilitated pulmonary uptake of ^{125}I–anti-ICAM, likely due to up-regulation of endothelial ICAM in response to cytokines. In rats, lipopolysaccharide (LPS) caused a 30% increase in the pulmonary uptake of ^{125}I–anti-ICAM (Figure 5), with a concomitant reduction in the blood level (pulmonary LR almost doubled from 50 to 85). In contrast, LPS suppressed pulmonary uptake of ^{125}I–anti-ACE in rats by 50% (pulmonary LR reduced from 14 to 7). Therefore, anti-ICAM targeting in LPS-treated rats was 10 times more robust than that of anti-ACE (LR 85 vs 7). Pulmonary targeting of ^{125}I–anti-ICAM was stably enhanced at 5 and 24 hours after LPS injection (LR 77 and 85). A similar elevation of

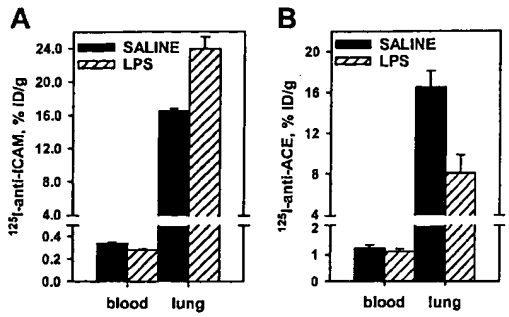

Figure 5. Endotoxin enhances ^{125}I–anti-ICAM pulmonary targeting. ^{125}I–anti-ICAM (left panel) or ^{125}I–anti-ACE (right panel) was injected in control rats (black bars) or after intraperitoneal injection of LPS (hatched bars). Lung and blood level of ^{125}I was determined 1 hour later. Data are presented as means ± SEM, n = 4.

^{125}I–anti-ICAM pulmonary targeting was seen in LPS-treated mice (not shown). The ^{125}I–anti-ICAM pulmonary uptake was doubled in mice exposed to 98% O_2 atmosphere, in contrast with a 50% decrease in the ^{125}I–anti-TM uptake (not shown). Therefore, proinflammatory factors suppress anti-ACE and anti-TM but augment anti-ICAM targeting.

Visualization of ICAM-directed vascular immunotargeting in animals

We visualized the pulmonary localization of anti-ICAM in mice by electron microscopy. Specific binding of the secondary gold-labeled antibody was evident in lungs harvested 4 hours after anti-ICAM injection (Figure 6A). Semiquantitative analysis after anti-ICAM injection revealed 16 ± 3 endothelium-associated particles/field versus 3 ± 1 particles/field associated with alveolar epithelium and interstitium (M ± SEM, 10 fields). Anti-ICAM was primarily localized along the luminal surface of the endothelium (arrows in Figure 6Ai). Noteworthy, we did not see endocytic vacuoles containing gold particles, the hallmark of endothelial uptake of internalizable conjugates in the lungs.[26]

To test whether anti-ICAM delivers an active enzyme cargo to endothelium, we conjugated a reporter enzyme, β-galactosidase, with anti-ICAM or control IgG. Figure 6B shows the results of X-Gal staining of the organs 1 hour after injection of either anti-ICAM/β-Gal or IgG/β-Gal conjugate in mice. After IgG/β-Gal injection (Figure 6Bv-viii), β-Gal activity was seen in the peripheral zone of the splenic follicles, the known site of Fc-receptor mediated uptake of immunoconjugates.[26,33] The splenic follicles also were stained by anti-ICAM/β-Gal (Figure 6Bi), as

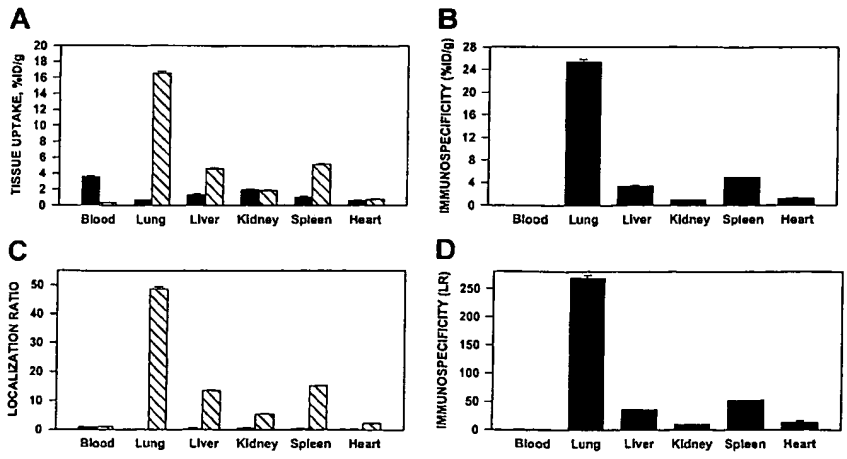

Figure 4. Pulmonary targeting of ^{125}I–anti-ICAM in rats. Biodistribution of ^{125}I–anti-ICAM (▨) or ^{131}I-IgG (■) 1 hour after intravenous injection in anesthetized rats. The data are shown as means ± SEM, n = 4. (A) Absolute values of the uptake in organs expressed as percent of injected dose per gram. (B) Immunospecificity index ($ISI_{\%ID/g}$), calculated as ratio of anti-ICAM to IgG %ID/g. (C) Localization ratio (LR) calculated as ratio of %ID/g in an organ to that in blood. (D) ISI_{LR} calculated as ratio of anti-ICAM LR to IgG LR.

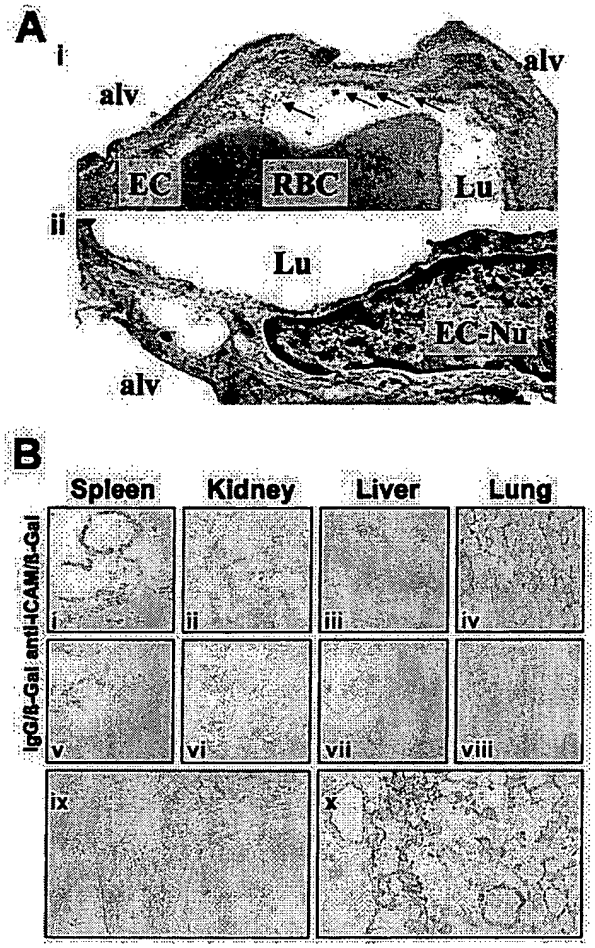

Figure 6. Localization of anti-ICAM and anti-ICAM/β-Gal in the pulmonary vasculature after injection in mice. (A) Immunogold electron microscopy of the lungs harvested 4 hours after intravenous injection of 100 μg anti-ICAM (i) or control IgG (ii). Arrows show endothelium-associated gold particles. RBC indicates red blood cells in a capillary lumen; Lu, vascular lumen; Alv, alveolar compartment; EC, endothelial cell; EC-Nu, endothelial cell nucleus. (B) Targeting of an active β-Gal conjugate was visualized 1 hour after injection in mice, using standard X-Gal chromogenic substrate staining protocol. Distribution of anti-ICAM/β-Gal (panels i-iv) and control IgG/β-Gal (panels v-viii). Detailed view of anti-ICAM/β-Gal in the lungs (panels ix and x). Original magnifications: Ai, × 70 000; Aii, × 60 000; Bi-Bviii, × 10; Bix-Bx, × 20.

were the renal glomeruli (Figure 6Bii), the known site of β-Gal elimination.[26,33]

However, injection of anti-ICAM/β-Gal, but not IgG/β-Gal, delivered β-Gal activity to the lungs (compare Figure 6Biv with Figure 6Bviii). Anti-ICAM/β-Gal was concentrated in the alveolar capillaries and in the lumen of larger vessels; no β-Gal activity was seen in subendothelial layers of blood vessels, interstitium, or airways (Figure 6Bix-x).

Effect of anti-ICAM conjugate size on endothelial internalization

Streptavidin was cross-linked to biotinylated anti-ICAM and biotinylated tPA at varying molar ratios of reactants, as previously studied with other proteins.[25-27] Analysis using dynamic light scattering analysis showed that conjugates ranging in size from 100 nm to several microns were generated, depending on the molar ratio between SA and biotinylated anti-ICAM (Figure 7A). Double staining of fluorescent-labeled anti-ICAM conjugates revealed that rat microvascular endothelial cells internalized those anti-ICAM conjugates having a diameter of 100-200 nm but did not internalize large conjugates around 1 μm (Figure 7B). In fact, after a 1-hour incubation at 37°C, the large, 1-2 micron double-labeled anti-ICAM conjugates (the preparation that corresponds to the peak farthest to the right in Figure 7A) decorated the entire cell surface (Figure 7C). Control IgG conjugates did not bind to endothelium irrespective of size and did not accumulate in the isolated rat lungs (not shown).

ICAM-directed targeting of tPA to the pulmonary vasculature

We then tested whether anti-ICAM would target an antithrombotic drug to sites of inflammation susceptible to thrombosis. To do so, we prepared large (approximately 1 μm), poorly internalizable anti-ICAM/^{125}I-tPA and IgG/^{125}I-tPA conjugates. One hour after intravenous injection, 7% of the injected anti-ICAM/^{125}I-tPA had accumulated in the rat lungs, compared with less than 0.3% for IgG/^{125}I-tPA (Figure 8A), a pulmonary $ISI_{\%ID/g}$ of 25 (Figure 8B). The blood level of anti-ICAM/^{125}I-tPA showed a corresponding decrease compared to IgG/^{125}I-tPA. Therefore, the pulmonary LR of anti-ICAM/^{125}I-tPA exceeded 20 (Figure 8C), with a calculated ISI_{LR} of approximately 80 (Figure 8D). Anti-ICAM/^{125}I-tPA was retained in the lungs for at least several hours after injection (not shown). Similar results were obtained in mice (not shown).

Anti-ICAM/tPA bound to the pulmonary endothelium surface retains plasminogen activity and dissolves intravascular clots

To test whether anti-ICAM can be used to deliver enzymatically active tPA to the endothelial lumen, we perfused anti-ICAM/tPA or

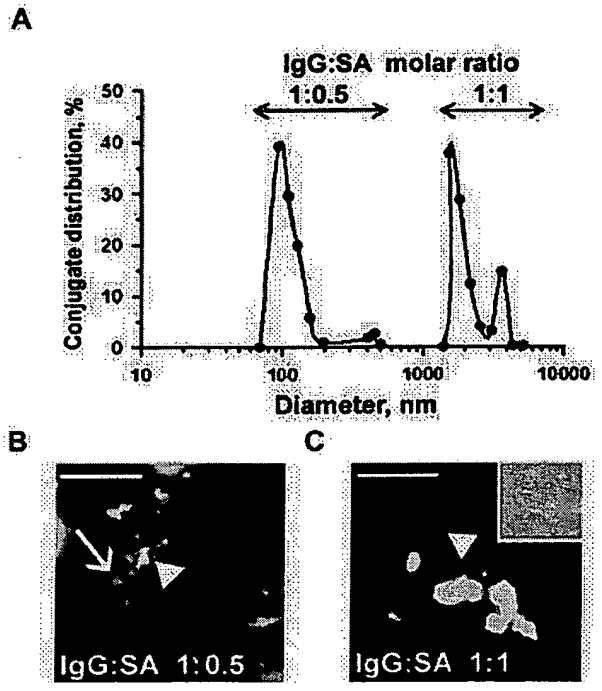

Figure 7. Size of anti-ICAM conjugates modifies their uptake by endothelial cells. (A) DLS analysis of size distribution of the conjugates prepared at molar ratio between biotinylated anti-ICAM and streptavidin of 1:0.5 (left peak) or 1:1 (right peak). (B-C) RPMVECs were incubated for 1 hour at 37°C with anti-ICAM conjugates containing rhodamine-labeled streptavidin with mean diameters of 100-200 nm (panel B) or larger than 1 μm (panel C). The surface-bound fraction of the conjugate was double-labeled using an FITC-labeled secondary antibody. Red color (arrows) denotes internalized conjugates; yellow color (arrowheads) denotes the noninternalizable, larger conjugates. White bars in panels B and C correspond to 5 μm size. Panel C inset shows the contrast phase micrograph (× 40) minimized to ⅛ the original size.

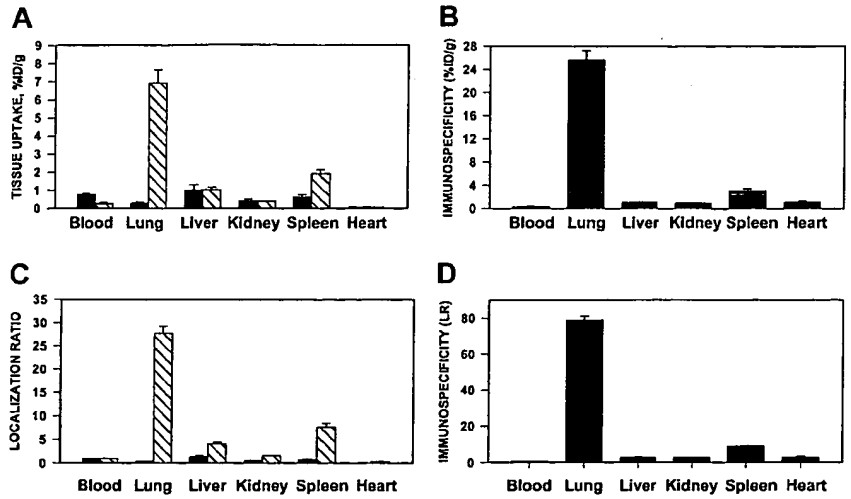

Figure 8. Pulmonary targeting of ^{125}I-tPA conjugated with anti-ICAM in rats. Biodistribution of ^{125}I-tPA conjugated to anti-ICAM IgG (▨) or control IgG (■) 1 hour after intravenous injection in anesthetized rats. The data are shown as means ± SEM, n = 4. (A) Absolute values of the uptake in organs expressed as percent of injected dose per gram. (B) Immunospecificity index ($ISI_{\%ID/g}$), calculated as ratio of anti-ICAM to IgG %ID/g. (C) Localization ratio (LR) calculated as ratio of %ID/g in an organ to that in blood. (D) ISI_{LR} calculated as ratio of anti-ICAM LR to IgG LR.

IgG/tPA in IPL. In all experiments, the vasculature was washed free of unbound conjugates prior to measuring tPA uptake and activity. Anti-ICAM but not the control IgG carriage led to pulmonary accumulation of ^{125}I-tPA (Figure 9A).

Aliquots of lung homogenates obtained after perfusion of anti-ICAM/tPA or IgG/tPA were then incubated with ^{125}I-fibrin clots at 37°C in vitro. Homogenates of lungs perfused with anti-ICAM/tPA caused 10-fold more fibrinolysis, measured by release of ^{125}I, than lungs perfused with IgG/tPA (Figure 9B).

We then infused a chromogenic tPA substrate into IPL. Enzymatic conversion of the substrate leading to appearance of a colored product was detected in the perfusate outflow of lungs preperfused with anti-ICAM/tPA but not those preperfused with IgG/tPA (Figure 9C). Therefore, the anti-ICAM/tPA associated with the luminal surface of the pulmonary endothelium retains its enzymatic activity.

Accessibility to a small synthetic substrate (mol wt < 500 D) does not prove that the anti-ICAM/tPA is accessible to convert its protein substrate, plasminogen. To examine this issue, a suspension of ^{125}I-microemboli was infused into the pulmonary artery 1 hour after perfusion with either anti-ICAM/tPA or IgG/tPA. The radioactivity in the lungs was determined 1 hour later as a measure of residual unlysed fibrin clots. Lungs preperfused with anti-ICAM/tPA practically completely dissolved the radiolabeled fibrin clots, whereas fibrinolysis in the lungs perfused with IgG/tPA did not differ significantly from the basal level measured in control lungs (Figure 9D). Therefore, anti-ICAM/tPA accumulates in the lungs, resides in enzymatically active form on the luminal endothelial surface, and thereby markedly facilitates fibrinolysis in the pulmonary vasculature.

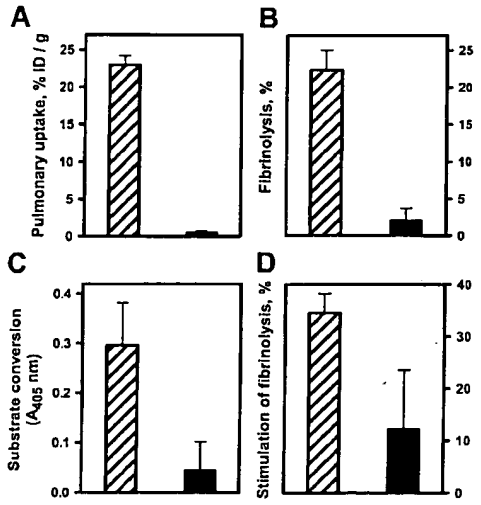

Figure 9. Anti-ICAM/tPA accumulated in pulmonary vasculature facilitates fibrinolysis. Isolated rat lungs were perfused with anti-ICAM/tPA (▨) or IgG/tPA (■) for 30 minutes at 37°C and washed free of unbound conjugates (5 minutes of noncirculating perfusion with a buffer). (A) Pulmonary uptake of ^{125}I-tPA conjugated to either anti-ICAM or control IgG. (B) Fibrinolysis of fibrin clots by aliquots of lung homogenates obtained after perfusion. (C) Conversion of chromogenic tPA substrate perfused after the conjugates. (D) Dissolution of radiolabeled fibrin emboli lodged in the pulmonary vasculature after perfusion of the conjugates. The data are shown as means ± SEM, n = 4.

Discussion

Drug targeting and concurrent blocking of a noninternalized highly expressed pro-inflammatory determinant expressed on the endothelial lumen that is stably up-regulated in the perturbed vasculature may provide a specific and powerful approach for treatment and prophylaxis of vascular inflammation and thrombosis. Our data indicate that ICAM-1 (CD54) fulfills the criteria of an "ideal" target for this specific goal and that anti-ICAM may be used for vascular immunotargeting of antithrombotic drugs.

Static human endothelial cells cultured under sterile conditions constitutively express relatively modest levels of ICAM-1 (Figure 1). However, the level of expression is much higher in vivo,[34] and anti-ICAM binds to the resting endothelium in intact animals.[20,34] Diverse cell types express ICAM-1, but the largest fraction directly accessible to the bloodstream is exposed on the endothelial surface.[34] This fact explains anti-ICAM targeting in vascularized organs (Figures 2 and 4) and confinement of the targeted cargoes to the vascular lumen (Figures 2, 6, and 9).

The pulmonary vasculature is the first major capillary network encountered by intravenously injected antibodies, contains roughly one-third of the endothelium in the body, is exposed to the entire cardiac output of venous blood, and, therefore, comprises the preferred target for affinity carriers recognizing pan-endothelial determinants.[28,31] Importantly, pulmonary uptake of anti-ICAM and anti-ICAM conjugates is not due to a nonspecific binding or mechanical retention in the vasculature, as control IgG counterparts neither bound to HUVECs nor accumulated in the lungs. In fact, the immunospecificity of the anti-ICAM and anti-ICAM/tPA conjugate pulmonary accumulation in normal rats approaches

values of 250 and 50, respectively (Figures 4 and 8). Analysis of the quantitative binding data obtained in rat lungs (Figure 2) indicates that binding of approximately 5-50 mg anti-ICAM can be expected in the human pulmonary vasculature. Thus, anti-ICAM carriers are likely to provide robust and preferential targeting to the pulmonary endothelium in intact animals, matching the characteristics of the best candidate carriers tested to date, including antibodies directed against PECAM, ACE, and a caveoli-associated antigen gp90.[15,24,28,31,33]

Certain pathological conditions suppress targeting to other constitutive endothelial determinants, such as thrombomodulin and ACE.[18,35,36] For example, endotoxin inhibits anti-ACE targeting in rats by 50% (Figure 5). In contrast, cytokines, oxidants, abnormal shear stress, and thrombin[37] are all known to enhance endothelial ICAM-1 expression[34,38] and augment anti-ICAM vascular targeting in vivo.[16,20] Up-regulation of endothelial ICAM-1 expression by thrombin[37] also makes it a preferred candidate for delivering antithrombotic agents. Our data extend these observations and reveal that (1) cytokine stimulation does not augment anti-ICAM internalization (Figure 3) and (2) pulmonary targeting of anti-ICAM is stably augmented in models of proinflammatory challenge in vivo (eg, Figure 5). This feature distinguishes targeting ICAM from targeting selectins, which are only transiently exposed on the perturbed endothelium.[39]

Antibodies and conjugates may unintentionally suppress important functions of endothelial proteins with potentially deleterious consequences (eg, thrombosis), making them less suitable for the therapeutic targeting.[40] ICAM-1, a counter-receptor for leukocyte integrins, supports cell adhesion on endothelium.[34,41] Because anti-ICAM suppresses inflammation by blocking leukocyte adhesion,[34,38,41-44] drug targeting to endothelial ICAM-1 is unlikely to have unintended deleterious effects on the host and, indeed, may provide secondary therapeutic benefits against inflammation, thrombosis, and oxidative stress. This feature of anti-ICAM conjugates deserves additional investigation.

Published studies on anti-ICAM internalization have yielded inconsistent results: epithelial and blood cells have been reported to internalize ICAM ligands in vitro,[45,46] but fragmentary data in other cell types showed the opposite outcome.[47,48] Our studies in cell culture, perfused rat lungs, and in animals show that endothelial cells internalize ICAM antibodies poorly (Figures 1, 2, 3, and 6). Thus, ICAM seems to be well suited for drug targeting to the luminal surface. This feature distinguishes ICAM from other similarly prevalent endothelial determinants, all of which are rapidly internalized, including thrombomodulin and ACE (Figures 1 and 2), selectins,[47-50] and caveoli-associated antigens.[15] A monoclonal antibody against gp85 antigen accumulates in the rat lungs and is not internalized and therefore can be used for surface targeting in this species,[29] but the identity, function, and regulation of its human counterpart are not known.

The uptake of anti-ICAM conjugates is modified by their size: endothelium internalizes conjugates with a mean diameter of 100-200 nm, but not anti-ICAM conjugates larger than 1 μm (Figure 7). This result indicates that anti-ICAM follows the paradigm observed previously with antibodies directed against another noninternalizable determinant, PECAM-1.[26-28] Conjugate size can be readily and stably modulated by varying the molar ratios of the reactants, as measured by dynamic light scattering (Figure 7). In theory, therefore, anti-ICAM represents a carrier that can be modified to facilitate drug delivery to either the endothelial surface (using monomolecular conjugates or conjugates larger than 500 nm) or to the intracellular compartment (using 100-300 nm conjugates). The anti-ICAM/tPA conjugates used in the present study were around 1 μm in size, which exceeds the effective internalizable size. Clearly, anti-ICAM/tPA bound along the cell surface retains enzymatic activity in the pulmonary vascular lumen and augments intravascular fibrinolysis (Figure 9).

This result serves as a proof-of-principle that ICAM represents a suitable target to deliver antithrombotic agents to the endothelial lumen. Although anti-ICAM/tPA conjugate itself may represent a useful agent, we anticipate that the strategy can be further optimized by generation of a monomolecular noninternalizable anti-ICAM/tPA fusion protein.

To our knowledge this is the first study showing that fibrinolytic agents can be used in a prophylactic mode to protect vital vascular beds, including those in the lungs. Patients are at high risk to develop pulmonary emboli after trauma: (1) when extensive proximal deep venous thrombi are present; (2) after recent pulmonary emboli; (3) in the setting serious respiratory compromise due to diverse cardiopulmonary disease; or (4) when the risk of bleeding after systemic anticoagulation is prohibitive.[1,2] Our data suggest that targeted delivery of antithrombotic agents to the pulmonary endothelium itself may be a suitable alternative in some of these settings, although additional studies will be needed to establish the duration and extent of fibrinolytic activity that is delivered.

In summary, ICAM possesses a number of highly desirable characteristics as a target for antithrombotic and anti-inflammatory drug delivery. Anti-ICAM targeting may allow a spectrum of novel therapeutic approaches, for example, a strategy to facilitate the antithrombotic potential of the pulmonary vasculature in patients at high risk to develop acute lung injury (ALI/ARDS) and thromboembolism. Future studies in larger animals will define potential therapeutic applicability and limitations of this strategy.

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